Award Number: W81XWH-06-1-0294

TITLE: Mammary Gland Tumor Development in Transgenic Mice Overexpressing Different Isoforms of the CDP/Cux Transcription Factor

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REPORT DATE: March 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;
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**Title:** Mammary Gland Tumor Development in Transgenic Mice Overexpressing Different Isoforms of the CDP/Cux Transcription Factor

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Fort Detrick, Maryland 21702-5012

**Abstract:**
Short CUX1 isoforms were found to be overexpressed in breast cancer cell lines, in human breast tumors and in uterine leiomyomas, suggesting that these proteins play a key role in tumor development and progression. My project consisted in analyzing the effect of these CUX1 isoforms on mammary gland development and tumorigenesis. Also, I worked on the identification of targets of CUX1 mediating its oncogenic properties. So far, I have shown that overexpressing short CDP/Cux isoforms leads to abnormal development of the mammary gland. Furthermore, overexpressing p75, p110 or p200 CDP/Cux leads to the development of mammary gland tumors in mice. These tumors seem to be of basal origin, suggesting that CUX1 promotes tumorigenesis in a precursor cell. Breast tumor patients with similar types of disease have very low chances of survival, since no specific treatment is currently available for them. Thus, my research project will enable us to gain a better understanding of the biological functions of each CUX1 isoform in mammary gland development and tumorigenesis, which could possibly lead to new therapeutic targets for the treatment of basal breast cancers.

**Subject Terms:**
Cancer, Breast Cancer, Oncogene, Proliferation, CDP/Cux, Transcription factor

**Security Classification:**
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INTRODUCTION

The CUX1 transcription factor is an important regulator of cell cycle progression, and was also found to be involved in many other processes, such as determination of cell-type identity, cell growth control, as well as cell migration and invasion (1-5). In addition, short CUX1 isoforms were found to be overexpressed in breast cancer cell lines, in human breast tumors and in uterine leiomyomas, suggesting that these proteins could play a key role in tumor development and progression (6, 7). I have also previously shown that overexpression of p75 CUX1 results in the development of myeloproliferative disease-like myeloid leukemia in mice (8). My project consisted in analyzing the effects of these CUX1 isoforms on mammary gland development and tumorigenesis, which allowed us to demonstrate the oncogenic role of CUX1 on mammary epithelial cells. I also worked on the identification of targets of CUX1, the deregulation of which could lead to cancer progression. In this report, I will describe the mammary gland tumors developed in the CUX1 transgenic mice.

BODY

1- Determine the effect of overexpression of the CUX1 isoforms on mammary gland development (Task 1)

Generation of MMTV-LTR/p75 and p110 CUX1 transgenic mice

To assess and compare the oncogenic potential of p75 and p110 CUX1, we set out to generate transgenic mice that would express these isoforms specifically in mammary epithelial cells. The transgenes contained coding sequences for p75 or p110 CUX1 downstream of the long terminal repeat of the Mouse Mammary Tumor Virus (MMTV-LTR). To avoid complications resulting from variations in copy number and integration site effects, we used the method of targeted transgenesis to insert the construct into the mouse hypoxanthine phosphoribosyltransferase (hprt) locus (9). After 8 backcrosses into the FVB strain of mice, females were made multiparous.

Transgene expression was monitored by RT-PCR and immunohistochemical staining, and exhibited the pattern usually observed with MMTV-LTR driven transgenes. Expression was elevated at 5 weeks of age, returned to a low level at 3 months in virgin mice, and later increased during early pregnancy and lactation (Appendix 1B and C). Note that immunohistochemical staining with the CUX1 antibodies was not sensitive enough to detect expression of the endogenous CUX1 protein in wild-type mice (Appendix 1C, top panels). We conclude, therefore, that the staining observed in transgenic mice must reflect transgene expression (Appendix 1C). A confirmation of this notion was provided from the comparison of results obtained with the 861 and 1300 CUX1 antibodies. While staining was observed in both the p75 and p110 CUX1 transgenic mice with the 1300 CUX1 antibodies, as predicted staining was seen only in the latter mice with the 861 CUX1 antibodies that recognize an epitope present only in the p110 isoform (Appendix 1C, compare 7.5P 1300 and 861 in p75 and p110 CUX1 mice). Note that the Hprt locus is situated on chromosome X. Therefore, as a result of random inactivation of one X chromosome in each cell, transgenes would be expected to be expressed in approximately 50% of the cells in females. In practice, however, transgene expression was observed in
less than 50% of mammary epithelial cells (Appendix 1C and data not shown).

**Ductal outgrowth at 5 weeks of age in p75 CUX1 transgenic mice**

Mammary gland whole-mounts were prepared from wild-type, p75 CUX1 and p110 CUX1 transgenic mice at 5 weeks and 3 months of age. At 5 weeks, a faster ductal outgrowth was observed in the p75 CUX1, but not in the p110 CUX1 transgenic mice (Appendix 1D). At 3 months, no difference was noted between wild-type and transgenic mice, in agreement with the observed low transgene expression at that time (Appendix 1D).

**2- Determine the effect of overexpressing CUX1 on mammary gland tumorigenesis in mice having reached a pure genetic background (Task 2)**

**MMTV-p75 and p110 CUX1 transgenic mice develop late-onset mammary carcinomas**

Cohorts of multiparous p75 CUX1 (n=66), p110 CUX1 (n=74) and wild-type FVB mice (n=88) were monitored for tumor incidence over 2 years. Tumors were detected in many organs and tissues, and overall tumor incidence was 32% and 53% in p75 and p110 CUX1 transgenic lines, respectively, as compared to 22% in wild-type FVB/N mice (Appendix 2A). Mammary tumors developed with an average latency of 20.5 months in 17% and 12% of p75 and p110 CUX1 transgenic lines, respectively, as compared to 3% of wild-type FVB/N mice (Appendix 2A). In summary, higher CUX1 expression is associated with increased incidence of mammary tumors.

**Mammary tumors are of diverse histological types**

Histopathological analysis revealed that mammary tumors were of diverse histopathological types (Appendix 2B-H). Some mammary tumors were classified as solid carcinomas with or without papillary differentiation (Appendix 2B, C), adenosquamous carcinomas (Appendix 2D, E), adenomyoepithelioma (Appendix 2F) or tubular/acinar adenoma (Appendix 2G). Interestingly, adenosquamous carcinomas were most common in the p75 CUX1 line (73% of all breast tumors), whereas solid carcinomas were most common in the p110 CUX1 line (56% of breast tumors) (Appendix 2H). Two p110 solid carcinomas displayed papillary differentiation, and interestingly, one of the two also contained areas of adenosquamous differentiation. One p75 solid carcinoma was described as being acinar, while another one was undifferentiated; in the same mouse, an adjacent mammary gland also contained multifocal squamous nodules. One p75 mouse developed mammary intraepithelial neoplasia (MIN), which was not observed in any control mouse (data not shown).

Immunohistochemical staining for cytokeratin 6, 14 and 8/18 confirmed the heterogeneity in histological phenotypes (Appendix 3). All tumors included a population of cells staining immunopositive for CK8/18, a marker of luminal epithelial cells (Appendix 3C, F, I, L). In addition, in most tumors we observed populations of cells immunopositive for CK14, a marker of myoepithelial cells (Appendix 3B, H, K). Moreover, the presence in some tumors of many cells immunopositive for CK6, in
particular in adenosquamous carcinomas and in the adenomyoepithelioma, suggested that these tumors also contained a proportion of progenitor cells (Appendix 3A and G).

**The p75 CUX1 isoform stimulates cell migration and metastasis to the lung**

Metastasis to the lung was observed in two p75 CUX1 transgenic mice with a primary solid carcinoma (Appendix 4A). One primary carcinoma was undifferentiated (Appendix 4A, left panel), while the other one was of the acinar type (Appendix 4A, right panel). The fact that metastasis to the lung was observed only in the p75 CUX1 transgenic mice led us to consider the possibility that this particular isoform of CUX1 could stimulate cell motility. To test this notion, we introduced a retrovirus expressing p75 CUX1 in mammary epithelial NMuMG<sup>NYPD</sup> cells and monitored the migratory capability of these cells in a two-chamber migration assay and a wound-healing assay. In both assays, cells expressing p75 CUX1 migrated faster than control cells carrying an empty vector (Appendix 4B). We then performed a tail vein assay in nude mice and after 90 days mice were sacrificed and examined for the presence of metastases in the lung. Approximately twice as many metastases were observed, and the total surface area covered by lung metastases was also approximately twice as large, in mice injected with cells expressing p75 CUX1, but the differences were not quite significant due to the variability in the number of metastases in individual mice (Appendix 4C). Together these results demonstrate that p75 CUX1 can stimulate cell migration in tissue culture assays and appears to increase moderately the ability of cells to invade the lung tissue.

**Mammary tumors express an active CUX1 transgene**

The CUX1 transgene was found to be expressed at the RNA level in all mammary tumors and in most of the adjacent normal mammary glands (Appendix 5A). In many cases, transgene expression was higher in the tumor than in the adjacent normal mammary gland.

From immunohistochemical staining, proteins produced by the CUX1 transgenes were detected in the nucleus of tumor cells (Appendix 5B). In some tumors, notably in adenosquamous carcinomas, a clear signal was observed in only a small fraction of tumor cells (Appendix 5B, rightmost panel). Due to the low sensitivity of this assay as noted before (Appendix 1C), we were unable to determine whether the transgene was expressed in only a fraction of cells or in all tumor cells but often below detection level. Since transgene expression was detected in fewer cells in adenosquamous carcinomas, we consider it likely that the MMTV regulatory sequences were silenced as a result of metaplasia, as observed by other groups (10, 11).

Immunoblotting analysis showed that CUX1 protein expression also was higher in the tumor than in the adjacent normal mammary gland (Appendix 5C). In agreement with this, CUX1 DNA binding activity was higher in tumors than in normal adjacent tissues (Appendix 5C).

In some cases, we noted that CUX1 DNA binding activity appeared relatively higher than what was predicted from the level of protein expression. To verify this observation and confirm it via an alternative approach, we performed in parallel immunoblotting and South Western analysis using as a probe double-stranded oligonucleotides containing the CUX1 consensus binding site. In these experiments, we purposely lowered the amount of tumor proteins in order to ensure that there would be equal or even lower amount of
CUX1 protein in the tumor sample than in the normal tissue sample. As can be seen in Appendix 5D, DNA binding was much stronger in tumor than in normal tissue samples. Importantly, as the South Western assay involves the separation of proteins in the presence of SDS and their subsequent renaturation, the DNA binding activity that is observed reflects the intrinsic activity of the protein independently of its interactions with various partners in cells. These results suggest that changes in post-translational modifications can contribute to increase the activity of CUX1 transgenes in tumor cells.

**ErbB2 overexpression cooperates with CUX1 in the formation of solid carcinomas**

Previous studies in transgenic mice have shown that particular oncogenes tend to cause mammary tumors of a definite histological type. For example, the MMTV-ErbB2 transgene will cause the formation of solid carcinomas, whereas activation of the Wnt pathway will lead to the formation of tumors that display myoepithelial, acinar, or glandular differentiation (12). We considered the possibility that different oncogenic pathways cooperated with CUX1 in the development of mammary tumors of distinct histological types. To begin to assess this possibility, we analyzed the expression of erbB2 in solid carcinomas and Wnt genes in adenosquamous carcinomas from CUX1 transgenic mice. Remarkably, erbB2 mRNA and/or protein expression was elevated in most solid carcinomas tested (Appendix 6A). As shRNA-mediated knockdown of CUX1 did not cause a reduction in erbB2 expression in breast tumor cells (data not shown), we concluded that the increase in erbB2 expression was not induced by CUX1 but rather was caused by molecular events that are independent of CUX1.

**CUX1 contributes to activate the Wnt/β-catenin pathway in adenosquamous carcinomas**

In contrast to the situation in solid carcinomas, in most adenosquamous carcinomas we observed an elevation in the expression of a Wnt gene: Wnt6 and Wnt10A were overexpressed in 5 tumors out of 6, whereas Wnt1 and Wnt8B were overexpressed in 2 tumors (Appendix 6B, top panel). In agreement with these results, 3 out of 3 adenosquamous carcinomas scored immunopositive for β-catenin in the nucleus, whereas no signal was detected in the solid carcinomas tested (Appendix 6B, lower panel). The role of CUX1 in the regulation of Wnt gene expression was first suggested from the decrease in Wnt mRNA levels following shRNA-mediated knockdown of CUX1 in breast tumor cells (Appendix 6C, top panel). Chromatin immunoprecipitation followed by quantitative real-time PCR analysis demonstrated that CUX1 can bind to the promoters of Wnt1, Wnt6, Wnt8b and Wnt10A (Appendix 6C, lower panel). In reporter assays, both p75 and p110 were able to activate expression from the Wnt1, Wnt6 and Wnt10a, but not the Wnt8b, gene promoters (Appendix 6D.). Altogether, these results indicate that CUX1 proteins bind to the promoters of several Wnt genes and contribute to their upregulation leading to the activation of the Wnt pathway, which culminates in the presence of β-catenin in the nucleus of mammary epithelial cells.

**p200 Transgenic Mice also Develop Mammary Gland Tumors**

Most of my work has focused on characterizing the mammary gland tumors developed in the p75 and p110 CUX1 transgenic mice. However, I observed that p200 CUX1 transgenic mice also developed mammary gland tumors with heterogeneous
histopathologies. This project will be continued by another student in our lab and will be the focus of another manuscript.

3-Generation of bi-transgenic mice overexpressing erbB2 and short CUX1 isoforms (Task 3)

Since our transgenic mice overexpressing the different CUX1 isoforms were found to develop tumors, I first focused on characterizing these tumors. Now that we know that erbB2 and CUX1 seem to collaborate in these mice in the induction of solid mammary gland tumors, it is more interesting to generate these bi-transgenic mice. This study is under way, but has so far not yielded any results. We have crossed erbB2 transgenic mice that develop tumors with long latencies of about 16 months with CUX1 transgenic mice that also develop tumors with long latencies. So it is normal that after a few months, our bi-transgenic mice still did not develop tumors. This task will be continued by another student in my laboratory.

4- Study the technique of specific transgenesis used to generate the MMTV transgenic mice through a comparison of the two different CUX1 p75 lines generated (Task 4)

In annual report 1, I established that the two lines expressed the transgene at the same locations at similar extent, so that the technique of site-specific transgenesis could be used to directly compare different transgenic mouse lines.
KEY RESEARCH ACCOMPLISHMENTS

- CUX1 is an oncogene

- Overexpressing various CUX1 isoforms in the mammary gland resulted in mammary gland tumorigenesis.

- Most CUX1 mammary gland tumors were adenosquamous or solid carcinomas.

- The transgenes were expressed in the tumors and displayed DNA binding activity.

- Some p75 CUX1 tumors metastasized to the lungs, whereas no p110 CUX1 tumors did.

- In a tissue culture system (NMuMg-NYPD cells), p75 CUX1 stimulated cell motility.

- p75 and p110 CUX1 were found to bind to the promoters of Wnt1, Wnt6, Wnt8b and Wnt10A.

- In adenosquamous tumors, β-catenin was stabilized in the nucleus, suggesting that activation of the wnt/β-catenin pathway collaborated with overexpression of CUX1.

- In solid carcinomas, upregulation of erbB2 seemed to cooperate with CUX1 overexpression.

REPORTABLE OUTCOMES

Manuscript

- **Chantal Cadieux**, Valérie Kedinger, Lu Yao, Maria Drossos, Marilène Paquet and Alain Nepveu, *MMTV-p75 and p110 CUX1 Transgenic Mice Develop Mammary Tumors of Various Histological Types*, Manuscript in revision at Cancer Research.

Abstracts


- **Cadieux C.**, Goulet B., Sansregret L., and Nepveu A. *The role of Short CDP/CUX in Cancer*, IABCR meeting, Montreal, October 2006.

- Cadieux C., Kedinger V., Harada R., and Nepveu A. Mammary Gland Tumor Development in Transgenic Mice Overexpressing Different Isoforms of the CUX1 Transcription Factor. Mammary Gland Biology Meeting, Barga, Italy, June 2008. (Refer to Appendix 7)

- Cadieux C., Kedinger V., Harada R., and Nepveu A. Mammary Gland Tumor Development in Transgenic Mice Overexpressing Different Isoforms of the CUX1 Transcription Factor. Era of Hope, Department of Defense Meeting, Baltimore, June 2008. (Refer to Appendix 7)

**Oral Presentations**

I have presented my work on various occasions to my colleagues:

- February 2006: The role of Short CDP/CUX in Cancer

- May 2006: Research Advisory Committee Meeting: CDP/Cux Transgenic Mice: The role of Short CDP/CUX Isoforms in Cancer

- May 2006: PhD proposal: The role of Short CDP/CUX Isoforms in Cancer

- February 2007: The role of CDP/CUX in Cancer

- September 2008: Research Advisory Committee Meeting: MMTV-p75 and p110 CUX1 Transgenic Mice Develop Mammary Tumors of Various Histological Types

- November 2008: Senior Seminar: MMTV-p75 and p110 CUX1 Transgenic Mice Develop Mammary Tumors of Various Histological Types
CONCLUSION

The p75 and p110 short isoforms of the CUX1 homeodomain protein are overexpressed in human breast tumors and breast cancer cell lines. To assess and compare the ability of these short CUX1 isoforms in driving mammary tumor development, we used site-specific transgenesis into the hprt locus to generate transgenic mice expressing p75 or p110 CUX1 under the control of the mouse mammary tumor virus long terminal repeat. We report that transgenic lines from both CUX1 isoforms developed mammary tumors of various histopathologies after a long latency period. Adenosquamous carcinomas and solid carcinomas were most common in the p75 and p110 CUX1 transgenic mice, respectively. Metastasis to the lung was observed in the p75 CUX1 transgenic mice only. Comparisons between tumors and adjacent normal mammary glands revealed that transgenes were overexpressed in most but not all tumors, yet in all cases tested CUX1 DNA binding was increased, suggesting that both higher expression and changes in post-translational modifications can contribute to stimulate transgene activity. Interestingly, higher expression of erbB2 mRNA was seen in most solid carcinomas, while adenosquamous carcinomas displayed higher expression of various Wnt genes and activation of the β-catenin pathway. Activation of erbB2 expression appeared to represent a cooperating event that occurred independently of CUX1. In contrast, chromatin immunoprecipitation, shRNA-mediated knockdown and reporter assays established that CUX1 proteins can bind to, and activate, the promoters of several Wnt genes. Together these results support the notion that oncogenic activity of CUX1 can facilitate the establishment of a Wnt/β-catenin autocrine loop.

“So what section”

My research has identified a new oncogene. I have provided evidence that overexpressing CUX1 contributes to the malignant transformation of epithelial cells in the mammary gland and thereby causes cancer in mice. This could allow identification of new therapeutic drug against breast cancer.

Furthermore, my research has enlightened the fact that overexpressing CUX1 seems to be associated with the development of tumors in mice that resemble a specific category of breast tumors called basal tumors in humans. These breast cancers are much harder to treat and often recur because we lack specific molecules to target to kill these cancer cells. My research has possibly identified one of these targets.
REFERENCES

Appendix 1: Expression of CUX1 transgenes in the mammary gland during development

(A) The p75 and p110 CUX1 coding sequences, under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR), were introduced by specific transgenesis into the Hprt locus.

(B) Expression of p75 and p110 CUX1 transgenes in the mammary gland was analyzed by reverse-transcription polymerase-chain-reaction (RT-PCR) at 5 weeks (virgin), 3 months (virgin), 7.5 day pregnancy (P), 6 day lactation (6L) and 4 day involution (4I). Primers used are shown by arrows in (A).

(C) Immunohistochemical staining of mammary glands in 5 week old virgin, 3 month old virgin and 7.5 day pregnant mice. Results are shown for wild-type, p75 and p110 CUX1 mice. The epitopes recognized by the 861 and 1300 CUX1 antibodies are shown in (A).

(D) Whole-mount analysis of mammary gland development in virgin mice at 5 weeks and 3 months of age (left panel). Quantification of the enhanced ductal outgrowth observed in p75 CUX1 mice at 5 weeks of age (right panel). *, p-value<0.05.
A

hprt locus       MMTV       CUX1       hprt locus
oligo1   oligo2

\[
\begin{array}{c}
p75 \\
p110
\end{array}
\]

CR2 CR3 HD R1 R2

p75

\[
\begin{array}{c}
861 \\
1300
\end{array}
\]

B

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<th>Transgene</th>
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<td>p75 mice</td>
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<td>p110 mice</td>
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C

5 weeks 1300  | 3 months 1300  | 7.5P 1300  | 7.5P 861
Wild-type
p75 CUX1
p110 CUX1

D

5 weeks  | 3 months
Wild-type
p75 CUX1
p110 CUX1

Ductal Outgrowth at 5 Weeks

Cadieux et al., App.1
Appendix 2: p75 and p110 CUX1 transgenic mice develop mammary gland tumors with various histopathologies.

(A) Tumors arising in various tissues of p75 CUX1, p110 CUX1 and wild-type mice. Other tumors include: ovarian tumors, Harderian gland adenoma of the eye, osteosarcoma and liver tumors. *, p-value≤0.05; **, p-value≤0.01.

(B-G) Histopathology of mammary tumors derived from p75 and p110 CUX1 transgenic mice show a spectrum of histological phenotypes, including solid carcinomas with (B) or without (C) papillary differentiation, adenosquamous carcinomas (D, E), adenomyoepithelioma (F), and tubular/acinar adenoma (G).

(H) Distribution of histopathological types in mammary tumors from p75 and p110 CUX1 transgenic mice.
### A

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<th>p75</th>
<th>p110</th>
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<tr>
<td>adenosquamous carcinoma</td>
<td>8 (73%)</td>
<td>2 (22%)</td>
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<tr>
<td>adenoma: tubular acinar</td>
<td>0</td>
<td>1 (11%)</td>
</tr>
<tr>
<td>adenomyoepithelioma</td>
<td>0</td>
<td>1 (11%)</td>
</tr>
<tr>
<td>solid carcinoma</td>
<td>3 (27%)</td>
<td>5 (56%)</td>
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### B

**Wild-type n=88**

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<th>39 (53%)**</th>
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<td>7 (8%)</td>
<td>10 (14%)</td>
<td>2 (3%)</td>
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<td>Mammary Gland Tumors</td>
<td>3 (3%)</td>
<td>9 (12%)*</td>
<td>11 (17%)**</td>
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<tr>
<td>Hematopoietic</td>
<td>1 (1%)</td>
<td>2 (3%)</td>
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<tr>
<td>Lung Tumors</td>
<td>10 (11%)</td>
<td>17 (23%)</td>
<td>8 (12%)</td>
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<tr>
<td>Other</td>
<td>2 (2%)</td>
<td>6 (8%)</td>
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**p110 n=74**

**p75 n=66**

### C

### D

### E

### F

### G

### H

**Type of mammmary tumor**

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<th>p110</th>
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<td>8 (73%)</td>
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<tr>
<td>solid carcinoma</td>
<td>3 (27%)</td>
<td>5 (56%)</td>
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Appendix 3: Different histopathological types of mammary tumors express distinct sets of cytokeratins.

Immunohistochemical staining of an adenomyoepithelioma (A-C), a solid carcinoma (D-F), an adenosquamous carcinoma (G-I), and an adenoma (J-L) for cytokeratin 6 (A, D, G, J), 14 (B, E, H, K) and 8/18 (C, F, I, L).
<table>
<thead>
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<th>Cytokeratin 14</th>
<th>Cytokeratin 8/18</th>
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<td>Adenomyo-epithelioma</td>
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<tr>
<td>CK6+, CK14+, CK8/18+</td>
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<td>Solid Carcinoma</td>
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<td>Adenoma</td>
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<td>CK6-, CK14+, CK8/18+</td>
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Appendix 4: Some mammary tumors from p75 CUX1 transgenic mice metastasize to the lungs

(A) H&E stainings of primary tumors and lung metastases

(B) Left Panel: Populations of NMuMG\textsuperscript{NYPD} mouse mammary epithelial cells stably expressing p75 CUX1 were submitted to two-chamber assays to evaluate their migration. Top cells were removed and the average pixel count was measured to evaluate the number of migrating cells. Representative results from at least three independent experiments are presented. **, pValue< 0.01.

Right Panel: Wound healing assay was performed with the same cells. Scratches were done on highly confluent cells and closure was monitored by taking pictures at different time points.

(C) Graphs and H&E stainings representing the average number of lung metastases or the total area covered by lung metastases per nude mouse injected with one million NMuMg\textsuperscript{NYPD} vector or p75 cells.
**A**

Mammary Tumor

Lung metastasis

Mammary Tumor

Lung metastasis

**B**

Migration Assay NMuMg_{NYPD}

![Graph showing pixel counts for Vector and p75 conditions.]

Scratch Assay NMuMg_{NYPD}

![Images of scratch assays at 0h and 7h.]

**C**

Number of Lung Metastases per Mouse

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<td>Mean</td>
<td>7.5</td>
<td>13.5</td>
</tr>
</tbody>
</table>

Area of Lung Metastasis per Mouse

<table>
<thead>
<tr>
<th></th>
<th>Vector</th>
<th>p75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.98</td>
<td>2.67</td>
</tr>
</tbody>
</table>

Cadieux et al., App.4
Appendix 5: Expression and DNA binding activity of CUX1 transgenes in mammary tumors.

(A) Expression of p75 and p110 CUX1 transgenes in mammary tumors and adjacent normal mammary glands was analyzed by reverse-transcription polymerase-chain-reaction. PCR-primers were designed to specifically amplify the transgenes. N, adjacent mammary gland; T, mammary gland tumor.

(B) Immunohistochemical staining of mammary tumors from p75 and p110 CUX1 mice, using 1300 CUX1 antibodies.

(C) CUX1 protein expression was analyzed by Western blotting using 1300 CUX1 antibodies, and DNA binding was assessed in electrophoretic mobility shift assays (EMSA) with double-stranded oligonucleotides containing a consensus binding site for CUX1.

(D) CUX1 protein expression was analyzed by Western blotting, and DNA binding was assessed by South Western blotting.
Appendix 6: Overexpression of erbB2 in solid carcinomas and activation of the wnt/β-catenin pathway in adenosquamous carcinomas.

(A) ErbB2 mRNA and protein expression was measured, respectively, by quantitative real-time PCR (top panel) and Western blot analysis (bottom panel) in solid carcinomas from p75 and p110 CUX1 transgenic mice.

(B) Expression of Wnt genes was measured by quantitative real-time PCR (top panel) and immunohistochemical staining for β-catenin (bottom panel) was performed in adenosquamous mammary tumors from p75 and p110 CUX1 transgenic mice.

(C) Expression of Wnt genes was measured by quantitative real-time PCR in Hs578T human breast tumor cells infected with CUX1 specific shRNA, treated or not with doxycycline (top panel), and the recruitment of CUX1 to Wnt genes was measured by chromatin immunoprecipitation in Hs578T (bottom panel).

(D) The promoters of the indicated Wnt genes were cloned into a luciferase reporter plasmid. Hs578T cells were transfected with each reporter plasmid together with a control vector or a vector expressing p75 or p110 CUX1. The experiments were done in triplicate and performed independently at least three times.
**A**

**ErbB2 Expression**

ErbB2 Expression

ErbB2 Expression

**B**

**Wnt genes Expression**

Wnt genes Expression

Wnt genes Expression

**C**

**Expression of genes in Hs578t cells with CUX1 knockdown**

Expression of genes in Hs578t cells with CUX1 knockdown

Expression of genes in Hs578t cells with CUX1 knockdown

**D**

**Blot: Ha**

Blot: Ha
Appendix 7

Chantal Cadieux, Valérie Kedinger, Ryoko Harada and Alain Nepveu

Mammary Gland Tumor Development in Transgenic Mice Overexpressing Different Isoforms of the CUX1 Transcription Factor

Abstract

The CUX1 transcription factor is involved in several processes including cell cycle progression, cell migration and invasion and the determination of cell-type identity. In non-transformed cells, the full-length protein of 200 kDa (p200 CUX1) is proteolytically processed by nuclear cathepsin L at the G1/S transition of the cell cycle into an isoform of 110 kDa (p110 CUX1). A second isoform of 75 kDa (p75 CUX1) is generated from an mRNA that is initiated within intron 20. Many studies suggest that the short CUX1 isoforms p75 and p110 are involved in cancer development. The p110 and p75 isoforms are overexpressed in primary human tumors, such as in uterine leiomyomas and breast cancers. Proteolytic processing of CUX1 is increased in many transformed cells and is no longer cell cycle regulated. In tissue culture, p110 and p75 stimulate cell proliferation by accelerating entry into S phase.

To investigate the oncogenic potential of CUX1, we engineered transgenic mice overexpressing p75, p110 or p200 under the control of the mouse mammary tumor virus promoter (MMTV). Each transgene was specifically integrated into the hypoxanthine phosphoribosyltransferase (hprt) locus and then backcrossed into the FVB background. Interestingly, in the three lines, 40 to 50% of mice developed tumors by the age of 24 months, which is significantly above the wild-type level of 19%. Tumors arose mainly in the mammary gland, the uterus and the lungs. Mammary gland tumors in the three lines were highly heterogeneous, with some being solid carcinomas, others containing areas of squamous metaplasia or papillary differentiation and others being more glandular.

In many tumors, the transgene was found to be overexpressed and activated. Experiments performed with cell lines established from the CUX1 tumors revealed that inhibition of CUX1 reduced the migration capacity of those cells. Tumors from the p200 CUX1 transgenic mice were found to overexpress cathepsin L and p110 CUX1, and to display enhanced p110 activity. These findings suggest that increased proteolytic processing of p200 CUX1 could drive tumor development in this mouse line. Finally, from ChIP-on-chip analyses in the Hs578t cell line, several Wnt genes were identified as putative targets of CUX1, and some of these, such as wnt1 and wnt10A, were found to be overexpressed in the CUX1 mammary tumors.

In summary, this study provides evidence that CUX1 can act as an oncogene in breast epithelial cells and emphasizes the need to study and decipher the mechanisms of action of this transcription factor.