Proteolytic Processing of Cut Homeobox 1 by Neutrophil Elastase in the MV4;11 Myeloid Leukemia Cell Line

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Abstract
Proteolytic processing by cathepsin L generates p110 Cut homeobox 1 (CUX1) at the end of the G1 phase, whereas an alternative transcript encodes p75 CUX1. These short CUX1 isoforms were reported to be overexpressed in cancer cells, and transgenic mice overexpressing the p75 isoform were found to develop myeloproliferative disease—like myeloid leukemias.

In the present study, we report that the neutrophil elastase can also generate a short CUX1 isoform in the MV4;11 acute myeloid leukemia cell line. Proteolytic processing was so efficient that the full-length CUX1 protein was detected only when cells were maintained in the presence of the specific elastase inhibitor III. In agreement with these findings, higher levels of the processed cyclin E isoforms were also detected in MV4;11 cells. Reappearance of full-length cyclin E and CUX1 could be induced upon the treatment of MV4;11 cells with the differentiation inducer phorbol 12-myristate 13-acetate or, unexpectedly, following overexpression of a short recombinant CUX1 protein. In both cases, the mechanism involved transcriptional repression of the neutrophil elastase gene. This result revealed a negative feedback loop whereby CUX1 shuts down the expression of the protease that cleaves it. Overall, the findings in MV4;11 and other cancer cells suggest that various mechanisms are used in cancer to favor the expression of short CUX1 isoforms.

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Introduction
Proteolytic processing has emerged as an important posttranslational mechanism that modulates the subcellular localization and/or biochemical activities of proteins with functions as diverse as receptor, ligand, signaling molecules, and transcription factors. In the case of transcription factors, the consequence of proteolytic processing is often to redirect the localization of proteins from a cytoplasmic site to the nucleus (reviewed in refs. 1, 2). However, in some instances, the peptides generated by processing exhibit a half-life, biochemical properties, and biological functions that are distinct from that of their precursors (reviewed in ref. 3). For example, the promyelocytic leukemia–retinoic acid receptor α (PML-RARα) fusion protein was shown to be processed by neutrophil elastase (4). Myeloid expansion and delayed myeloid maturation could not be induced by PML-RARα expression alone but required, in addition, the activity of neutrophil elastase (5). Moreover, in transgenic mice carrying the PML-RARα fusion gene, inactivation of the neutrophil elastase gene conferred some protection against leukemia: Penetrance was reduced and latency was increased (4). Altogether, these findings suggested that the presence of neutrophil elastase in promyelocytes could explain why the expression of the PML-RARα fusion protein is invariably associated with the promyelocytic leukemia phenotype (4).

Cut homeobox 1 (CUX1) belongs to a family of transcription factors present in all metazoans and involved in the control of proliferation and differentiation (reviewed in ref. 6). The literature includes a variety of terms: Cut in Drosophila melanogaster, CCAAT displacement protein, and Cut-like (CUTL) in human and cux in mouse (7-10). The Human Genome Organization recently proposed to change from the gene root of CUTL (CUT-Like #) to CUX#. Thus, the term CUX1 will be used thereafter in the text to designate the human or mouse protein. Four CUX1 protein isoforms have been described thus far: p200, p150, p110, and p75. The full-length p200 protein carries the CCAAT displacement activity by binding rapidly but transiently to DNA, whereas p150 does not bind to DNA and may act as a dominant negative (11, 12). In contrast, the short p110 and p75 isoforms behave like classic transcription factors that engage in slow but stable interactions with DNA and can function as repressors or activators depending on promoter context (13-16). Although a tissue-specific transcript that is initiated within intron 20 encodes the p75 isoform, the p110 isoform is generated by a proteolytic processing event that is mediated by cathepsin L at the end of the G1 phase (13, 14, 17). At the cellular level, the p110 isoform was shown to stimulate cell proliferation and to accelerate entry into the S phase (18). These results extended early observations linking CUX1 to the control of gene expression in S phase (19, 20).

Microarray analysis revealed that CUX1 was one of the most up-regulated genes in malignant plasma cells from multiple myelomas compared with nonmalignant plasma cells (21).
Moreover, in a survey of human breast cancers, CUX1 expression was found to be significantly increased in high-grade carcinomas and was inversely correlated with survival (22). In uterine leiomyomas, p110 CUX1 was frequently expressed at a higher level in the tumor than in the adjacent myometrium (23). In many cancer cell lines, increased expression and activity of nuclear cathepsin L was found to result in a higher ratio of processed over the full-length CUX1 protein (24). Interestingly, the intron 20–mRNA and p75 isoforms were found to be expressed only weakly or not at all in primary human mammary epithelial cells and normal breast tissues, but were detected in many breast tumor cell lines and human breast tumors (13). In invasive breast tumors, a significant association was established between higher intron 20–mRNA expression and a diffuse infiltrative growth pattern (13).

Collectively, expression studies in primary tumors and cancer cell lines suggested that increased expression of CUX1, notably its short isoforms, could contribute to tumor development and/or progression. Indeed, transgenic mice engineered to express p75 CUX1 displayed enhanced susceptibility to various cancers. In particular, the p75 CUX1 mice frequently succumbed to a disease defined as a myeloproliferative disease–like myeloid leukemia and characterized by splenomegaly, hepatomegaly, and frequent infiltration of leukocytes into nonhematopoietic organs like the kidneys and lungs (25). The cell type of origin of these tumors came as a surprise because the transgene was placed under the control of the mouse mammary tumor virus long terminal repeat and was inserted into the mouse hypoxanthine phosphoribosyltransferase (Hprt) locus using the method of targeted transgenesis (26). The unexpected phenotype of transgenic mice suggested a particular tropism of the p75 CUX1 isoform toward myeloid cells. This led us to investigate CUX1 expression and activity in a panel of acute myeloid leukemia cell lines. We identified a cell line in which the full-length CUX1 protein seemed not to be expressed. Our analysis of CUX1 expression in the MV4;11 cell line revealed a novel mechanism by which short CUX1 isoforms can be generated.

**Results**

**The Full-length CUX1 Protein Is Not Detected in the MV4;11 Acute Myeloid Leukemia Cell Line**

We analyzed CUX1 expression in a panel of myeloid leukemia cell lines and found one cell line, MV4;11, which did not express the full-length p200 isoform but expressed only shorter isoforms. The full-length protein was easily detected in RS4;11 and U937 cells, but not in MV4;11 cells (Fig. 1A, lanes 1-3). Both the MV4;11 and RS4;11 cell lines derive from a myeloid leukemia that bears the 4;11 chromosomal translocation; we further compared CUX1 expression in these two cell lines by performing immunoprecipitation and immunoblotting with various CUX1 antibodies (27). Again, p200 was detected in RS4;11 and U937 cells, but not in MV4;11 cells (Fig. 1A, lanes 1-3). Both the MV4;11 and RS4;11 cell lines derive from a myeloid leukemia that bears the 4;11 chromosomal translocation; we further compared CUX1 expression in these two cell lines by performing immunoprecipitation and immunoblotting with various CUX1 antibodies (27). Again, p200 was detected in RS4;11 and U937 cells, but not in MV4;11 cells (Fig. 1A, lanes 1-3 and 8-10). Results from electrophoretic mobility shift assays were consistent with those from immunoblotting. In MV4;11 samples, we observed the slow migrating p200-complex that was supershifted with CUX1 antibodies (Fig. 1C, compare lanes with lanes 4, 8, and 12 with lanes 2, 6, 10, and 14). This complex was not detected in the MV4;11 samples; however, faster migrating complexes were observed (Fig. 1C, every odd number lane). These faster migrating complexes involved CUX1 protein species because incubation in the presence of various CUX1
antibodies generated distinct, albeit weak, supershifts (Fig. 1C, lanes 1, 5, 9, and 13). A pairwise comparison of the supershifts generated by each antibody revealed that some supershifts were unique to MV4;11, whereas others were unique to RS4;11 (Fig. 1C, compare lane 1 with 2, 5 with 6, 9 with 10, and 13 with 14). These findings suggest that MV4;11 and RS4;11 cells express distinct short CUX1 isoforms.

**MV4;11 Cells Express the Full-length p200 following Phorbol 12-Myristate 13-Acetate Treatment**

Analysis of CUX1 mRNA expression did not reveal any difference between the MV4;11 and RS4;11 cells (data not shown). Moreover, a DNA rearrangement involving the *CUTL1* locus at 7q22 was not detected in MV4;11 cells (ref. 27 and data not shown). Yet, we could not exclude that more subtle changes in DNA might have occurred in these cells. Convincing evidence that the gene was intact came from the observation that the full-length CUX1 protein was expressed in certain culture conditions. Upon treatment with phorbol 12-myristate 13-acetate (PMA), a commonly used differentiation inducer, MV4;11 cells stopped proliferating and started to adhere to the plate (Fig. 2A). Interestingly, with increasing amounts of PMA, we began to detect the full-length p200 CUX1 protein (Fig. 2B, compare lanes 1 to 4). This band was specific to CUX1 because it was competed out with the glutathione S-transferase/CUX1-861 immune peptide but not with the glutathione S-transferase peptide alone (Fig. 2B, compare lanes 1-4 with lanes 5-8 and 9-12). Of note, we did not detect changes in CUX1 mRNA expression upon treatment of MV4;11 cells with PMA (data not shown; see also Fig. 6A, lanes 2 and 3). These results suggest that DNA rearrangements or point mutations cannot account for the expression of short CUX1 isoforms in MV4;11 cells.

**A Recombinant Full-length CUX1 Protein Is Efficiently Processed in MV4;11 Cells**

One approach to verify whether proteolytic processing generates a short isoform is to express a recombinant full-length
protein using the entire coding sequence from the corresponding cDNA. We established stable populations of RS4;11 and MV4;11 cells carrying a retroviral vector that codes for a full-length CUX1 protein, herein called 1-1505. In MV4;11 cells, the full-length protein could now be detected, albeit at a much weaker level than in RS4;11 cells (Fig. 3A, compare lanes 1 and 3). Also, we observed an increase in a short CUX1 isoform that comigrated with an endogenous protein present in the parental MV4;11 cells (Fig. 3A, compare lanes 2 and 3; Fig. 3B, compare lanes 1 and 3). We interpret this band to represent a product of proteolytic processing. To confirm that MV4;11 cells cannot sustain elevated expression of the full-length CUX1 protein, MV4;11, 293 and HeLa cells were transiently infected with a retroviral vector expressing p200 CUX1 with a COOH-terminal hemagglutinin (HA) tag. In immunoblotting with an anti-HA antibody, the full-length CUX1 protein was easily detected in 293 and HeLa cells but not in MV4;11 cells (Fig. 3C, lanes 1-6). Yet, following treatment of MV4;11 cells with PMA, the protein was visible with the HA antibody (Fig. 3C, lane 8). Using the CUX1-861 antibody that recognizes an internal epitope revealed that addition of PMA to the stable population increased the steady-state level of the full-length CUX1 protein and reduced the amount of the processed isoform in both the parental and transduced MV4;11 cells (Fig. 3B, compare lanes 2 with 1 and 4 with 3). These findings confirmed that proteolytic processing generates a short CUX1 isoform in MV4;11 cells and that PMA treatment causes a decrease in processing.

Proteolytic Processing of CUX1 Using Nuclear Extracts from MV4;11 Is Inhibited by Serine Protease Inhibitors

We did in vitro processing assays using a bacterially expressed CUX1 protein as a substrate and nuclear extracts from RS4;11 and MV4;11 cells as a source of proteases. This assay was previously shown to reproduce in vitro the proteolytic processing of CUX1 (17, 28). Two novel cleavage products were generated when the CUX1 protein was incubated with nuclear extracts from MV4;11 cells (Fig. 4A, lane 3). This findings suggested that CUX1 might be proteolytically processed by a novel protease in MV4;11 cells. The assay was therefore repeated in the presence of various protease inhibitors. Interestingly, processing was not inhibited in the presence of E64, a cysteine protease inhibitor previously shown to inhibit CUX1 processing by cathepsin L in other cell lines (Fig. 4B, compare lane 3 with 2; refs. 17, 28). However, two serine protease inhibitors, leupeptin and phenylmethylsulfonyl fluoride, were able to inhibit processing by MV4;11 nuclear extracts (Fig. 4B, compare lane 2 with 3 and lanes 8 and 9 with 7). In contrast, as expected, phenylmethylsulfonyl fluoride did not inhibit processing when added to a reaction containing nuclear extracts from the He578T breast tumor cell line (data not shown). These results suggest that nuclear extracts from MV4;11 cells contain a serine protease that can cleave CUX1.

Expression of Neutrophil Elastase Is Elevated in MV4;11 Cells and Is Reduced upon PMA treatment

As p200 CUX1 was detected in PMA-treated MV4;11 cells, we reasoned that proteolytic processing of CUX1 might

![FIGURE 3.](https://example.com/figure3.png)

A recombinant full-length CUX1 protein is efficiently processed in MV4;11 cells. A, MV4;11 cells were stably infected with retroviral vectors expressing recombinant CUX1 proteins containing amino acids 1-1505 or 878-1505. Nuclear extracts were analyzed by immunoblotting using the 861 CUX1 and actin antibodies. Arrows, the recombinant proteins and the processed isoforms generated in MV4;11 cells. B, The MV4;11 cell lines stably expressing recombinant CUX1 proteins were treated or not with PMA for 48 h, and nuclear extracts were analyzed by immunoblotting as in A. C, MV4;11, 293 and HeLa cells were infected with a retroviral vector expressing the full-length CUX1 protein with a HA epitope tag at its COOH terminus. Lanes 6 and 7, MV4;11 cells were treated with 1 μmol/L PMA for 48 h. Nuclear extracts were analyzed by immunoblotting using antibodies against HA and actin.


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be reduced in these conditions (Fig. 2B). To confirm this hypothesis, the in vitro processing assay was repeated using as a source of proteases nuclear extracts from MV4;11 cells that were treated or not with PMA. Indeed, processing in vitro was reduced by the treatment of cells with PMA (Fig. 4C, lanes 2 and 3). As the substrate in this reaction was purified from bacteria, this result suggested that the effect of PMA was not mediated through modification of the substrate. Instead, PMA must affect the expression or activity of the protease in MV4;11 cells. We therefore investigated the effect of PMA on the expression of candidate proteases. RNA was isolated from RS4;11 and MV4;11 cells, treated or not with PMA, and protease mRNA expression was measured using a semiquantitative reverse transcription-PCR (RT-PCR) assay. In this assay, we were looking for a serine protease whose expression was higher in MV4;11 than in RS4;11 cells, and was reduced upon treatment with PMA. Expression of the neutrophil elastase mRNA fulfilled these criteria: The expression was much higher in MV4;11 than in RS4;11 cells; moreover, the expression was reduced in the presence of PMA (Fig. 4D, top). These results are in agreement with the previously reported transcriptional down-regulation of the neutrophil elastase gene upon treatment with PMA (29). Expression of cathepsin G mRNA, on the other hand, was not significantly different in the three samples (Fig. 4D, second panel). Interestingly, proteinase 3 mRNA expression was higher in MV4;11 than in RS4;11 cells, but its expression was not affected by PMA (Fig. 4D, third panel). Expression of cathepsin L mRNA was very weak in both cell lines but was induced in the presence of PMA (Fig. 4D, fourth panel). Indeed, PMA was previously shown to induce cathepsin L expression in various cell lines (30, 31). In summary, results from expression analysis revealed that neutrophil elastase was the only serine protease whose expression was both elevated in MV4;11 cells and reduced upon treatment of cells with PMA.

**FIGURE 4.** CUX1 is proteolytically processed by a serine protease in MV4;11 cells. A. In vitro processing assay. A recombinant CUX1 protein containing amino acids 612 to 1328 was expressed in bacteria, purified by affinity chromatography over nickel beads, and incubated in the presence of nuclear extracts (0.5 μg) isolated from RS4;11 or MV4;11 cells. The substrate and products of the reaction were analyzed by immunoblotting using anti-HA antibodies. *, contaminating protein present in the purified sample that is recognized by the HA antibody; note that this protein is not detected when the Myc antibody was used (see Fig. 3B). B. An in vitro processing assay was done as in A in the presence of the indicated protease inhibitors and analyzed by immunoblotting using anti-Myc antibodies. C. An in vitro processing assay was done as in A using nuclear extracts from MV4;11 cells that had been treated or not with 1 μmol/L PMA for 48 h. D. Total RNA was isolated from RS4;11, MV4;11 cells, and PMA-treated MV4;11 cells. RT-PCR analysis was done using primers specific for various hematopoietic serine proteases, cathepsin L, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**CUX1 Is Proteolytically Processed by the Human Neutrophil Elastase Serine Protease in MV4;11 Cells**

The in vitro processing assay was repeated this time using a specific inhibitor for neutrophil elastase, elastase inhibitor III. The amount of the two cleavage products was reduced in the presence of elastase inhibitor III (Fig. 5A, compare lanes 3 and 4 with lane 2). This result suggests that neutrophil elastase is the active protease in nuclear extracts from MV4;11 cells. Indeed, in vitro processing using a purified preparation of elastase generated two cleavage products of identical mobility (Fig. 5A, compare lane 7 with lane 10). Moreover, the addition of elastase inhibitor III to the reaction inhibited the production of cleavage products whether the reaction was done with purified neutrophil elastase or nuclear extracts from MV4;11 (Fig. 5A, compare lane 7 with 8, and lane 11 with 10). As elastase inhibitor III is a cell-permeable compound, we tested its effect when applied directly onto cells in tissue culture. Interestingly, the effect of elastase inhibitor III mimicked that of PMA. After 48 hours, the full-length CUX1 protein was detected (Fig. 5B, compare lane 5 with 2). Altogether, these results show that neutrophil elastase is overexpressed and cleaves CUX1 in MV4;11 cells.

**Cyclin E Is Also Proteolytically Processed by the Neutrophil Elastase in MV4;11 Cells**

Cyclin E has previously been identified as a target of elastase in breast cancer cells, and truncation of cyclin E was found to cause an increase in cyclin E/Cdk2 kinase activity (32, 33). We therefore predicted that cyclin E should be processed in MV4;11 cells. Indeed, the ratio of the full-length
over the processed cyclin E isoforms was much lower in MV4;11 than in RS4;11, Hs578T, and U937 cells (Fig. 5C, compare lane 3 with lanes 1, 2, and 6). Moreover, treatment of MV4;11 cells with either elastase inhibitor III or PMA reduced the amount of processed cyclin E and increased the amount of full-length cyclin E (Fig. 5C, compare lanes 4 and 5 with lane 3). These results prompted us to compare the expression levels of neutrophil elastase in MV4;11 and U937 cells. Neutrophil elastase mRNA expression was much higher in MV4;11 than in U937 cells (Fig. 5D, lanes 1 and 3). This finding is consistent with the processing pattern of cyclin E and CUX1 in these two cell lines (cyclin E, Fig. 5C, lanes 3 and 6; CUX1, Fig. 1A, lanes 2 and 3). We noted again that PMA caused a reduction in the expression of the neutrophil elastase but not of proteinase 3 mRNA (Fig. 5D, lanes 3 and 4). These results suggested that overexpression of neutrophil elastase is responsible for the enhanced proteolytic processing of both CUX1 and cyclin E in MV4;11 cells.

The Recombinant CUX1 878-1505 Protein Stimulates Expression of the Endogenous Full-length CUX1 Protein by Repressing Expression of the Neutrophil Elastase Gene

Infection of MV4;11 cells with a vector expressing a short recombinant CUX1 protein, called 878-1505, revealed an interesting phenomenon: The full-length p200 protein could now be detected in these cells (Fig. 3A, compare lanes 2 and 4; Fig. 3B, compare lanes 1 and 5). Further tests were done to confirm that these cells only carried the CUX1 878-1505 vector and were not mixed with other cells (data not shown). We investigated a number of mechanisms that could account for the induction of full-length CUX1 by a short recombinant CUX1 protein. RT-PCR analysis indicated that expression of endogenous CUX1 mRNA was not significantly increased in the MV4;11 cells that either carried the 878-1505 vector or were treated with PMA (Fig. 6A, lanes 2-5). However, we found that expression of the neutrophil elastase mRNA was reduced in MV4;11 cells that expressed the recombinant 878-1505 protein (Fig. 6A, compare lane 9 with 11). In agreement with this finding, processing of cyclin E was also reduced in MV4;11 cells expressing recombinant CUX1 proteins (Fig. 6B, compare lane 2 with 3 and 4). Altogether, these results reveal a negative feedback loop whereby CUX1 represses transcription of the neutrophil elastase gene, whereas neutrophil elastase cleaves the CUX1 protein.

Inhibition of Neutrophil Elastase in MV4;11 Cells Slows Down Cell Proliferation

To verify whether the activity of neutrophil elastase may confer a growth advantage to the MV4;11 cells, we measured cell proliferation in the presence of the elastase inhibitor III. Using two independent assays, cell counting and carboxyfluorescein diacetate succinimidyl ester staining, we observed a decrease in cell proliferation when MV4;11 cells were treated with the elastase inhibitor III (Fig. 7). These results are consistent with the notion that increased activity of neutrophil elastase in MV4;11 cells enhances their proliferation potential.

Discussion

The MV4;11 myeloid leukemia cell line represents the first case of cells in which the full-length CUX1 protein is not detected. Preliminary experiments as well as karyotyping
analysis did not provide any evidence of genomic DNA rearrangement or alternative mRNA expression in MV4;11 cells (27). Ultimately, the best evidence against the notion of DNA rearrangement or point mutations was the finding that MV4;11 cells could be induced to reexpress the full-length CUX1 protein when cultured in the presence of either PMA (Figs. 2B, 3B, and 5B) or the elastase inhibitor III (Fig. 5B), or when infected with a vector expressing CUX1 proteins (Fig. 6A, lanes 9-11). In agreement with these findings, cyclin E was processed by neutrophil elastase in MV4;11 cells in a manner similar to what was previously reported in other cancer cells (Figs. 5C and 6B; ref. 33). Proteinase 3 was also expressed at a higher level in MV4;11 than in RS4;11 cells, but the fact that its expression was not affected by PMA or CUX1 878-1505 argues against this protease being the main serine protease that cleaves CUX1 (Figs. 5D and 6A). Collectively, our data indicate that the predominant activity responsible for the proteolytic processing of CUX1 and cyclin E in MV4;11 cells is that of neutrophil elastase.

The proliferation of MV4;11 cells was reduced in the presence of elastase inhibitor III (Fig. 7). These results indicate that increased neutrophil elastase activity indeed confers a growth advantage to cells. Although it is likely that other substrates of neutrophil elastase also contribute to increase the proliferation potential of MV4;11 cells, several lines of evidence suggest that the efficient cleavage of CUX1 may have some functional significance. Proteolytic processing was shown previously to relieve the protein from the inhibitory effect of an NH2-terminal autoinhibitory domain (23, 24). In transgenic mice, forced expression of short CUX1 entry and stimulate cell proliferation (18). In contrast, the full-length protein did not have any effect in the same assays. 5 Whereas proteolytic processing of CUX1 was shown to be tightly regulated during cell cycle progression in normal cells, increased processing of CUX1 was reported both in primary human uterine leiomyomas and several cancer cell lines (23, 24). In transgenic mice, forced expression of short CUX1 proteins enhanced the susceptibility to various malignancies, in particular in the myeloid cell lineage (25). In contrast,

FIGURE 6. CUX1 represses expression of the neutrophil elastase gene. A. Total RNA was isolated from RS4;11, MV4;11, and MV4;11/878-1505 cells, treated or not with PMA. RT-PCR analysis was done using primers specific for CUX1, neutrophil elastase, proteinase 3, cathepsin G, and glyceraldehyde-3-phosphate dehydrogenase. B. Nuclear extracts from RS4;11 cells and MV4;11 cells stably expressing CUX1 1-1505 or 878-1505 were analyzed by immunoblotting using anti–cyclin E antibodies.

5 L.L. Sansregret and B. Goulet, unpublished observations.
a predisposition to cancer was not reported for the transgenic mice that express the full-length protein (34). Altogether, these results suggest that any event that leads to an increase in the level of short CUX1 isoforms is likely to provide cells with a proliferation advantage.

The findings reported in the present study add to the increasing evidence linking the aberrant activity of certain proteases with the altered localization or change in biochemical properties of proteins in cancer cells (33, 35-40). Cancer is often described as a genetic disease. However, nongenetic mechanisms also play a role in cancer. Like DNA mutations, proteolytic processing can lead to proto-oncogene activation and tumor suppressor inactivation depending on the domain that is cleaved off. Nongenetic changes in turn can lead to genetic changes that can be stably transmitted to daughter cells. Although aberrant proteolytic processing is much more difficult to assess than genetic changes because proteins are less stable molecules than DNA, it will be crucial in the future to establish a repertoire of cancers in which such mechanisms plays a role. The importance of this is not only conceptual but also practical, because serine and cysteine proteases are a type of enzyme for which it is feasible in practice to develop efficient and specific inhibitors that can be used as therapeutic agents.

**Materials and Methods**

**Cell Culture**

MV4;11 cells were grown in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum. RS4;11 and U937 were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were infected by the addition of virus containing supernatant from 293VSV producer cells in the presence of 8 \( \mu \)g/mL polybrene (Sigma). For PMA (Sigma) and elastase inhibitor III (Calbiochem) treatments, MV4-11 cells were plated at 5 \( \times \) 10^5/mL. Growth curves were done by plating in triplicate 2 \( \times \) 10^5 cells/35 cm, and viable cells were counted every day. Live cell images were taken with a Retiga 1300 digital camera (QIMAGING) and a Zeiss AxioVert 135 microscope (Carl Zeiss Canada).

**Carboxyfluorescein Diacetate Succinimidyl Ester Staining**

Cells were stained using the CellTrace CFSE Cell Proliferation Kit and were analyzed by flow cytometry with 488-nm excitation and emission filters appropriate for fluorescein, according to the manufacturer’s instruction (Molecular Probes). Carboxyfluorescein diacetate succinimidyl ester profiles were analyzed using the FlowJo software (Tree Star Software).

**CUX1 Antibodies**

To generate polyclonal antibodies \( \alpha \)1032 and \( \alpha \)1061, rabbits were injected with either 500 \( \mu \)g of purified bacterial fusion protein containing the regions of CUX1 (amino acids 1032-1106) or of the peptide MSSVESVK- COOH coupled to keyhole limpet hemocyanin (amino acids 1061-1069), in Freund’s complete adjuvant. Polyclonal antibodies were purified by affinity chromatography as previously described (14). The other antibodies used have been described (14).
**Immunoprecipitations**

Total protein extracts were prepared in modified radio-immunoprecipitation assay buffer [20 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 2 mmol/L EDTA, 2 mmol/L EGTA]. Lysates were precleared for 1 h with 40 μL protein A-Sepharose. Immunoprecipitation was done with the indicated antibodies overnight at 4°C. Protein A-Sepharose was then added for 1 h at 4°C. Immunoprecipitates were washed twice with wash buffer [20 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% SDS] and twice with wash buffer [20 mmol/L Tris (pH 8.0), 500 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% SDS] and resuspended in 2× SDS sample buffer.

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assays were done as previously described with 6 or 3 μg of nuclear extracts (14).

**Reverse Transcription-PCR**

RT-PCR was done as previously described (13).

**Purification of His-612-1328-HA-Myc**

pTriex2 vector encoding His-612-1328-HA-Myc proteins were introduced into the BL21(DE3) strain of *Escherichia coli* and induced with 1 mmol/L isopropyl-β-D-galactopyranoside for 3 h. Proteins were purified on Ni-NTA agarose and induced with 1 mmol/L isopropyl-β-D-galactopyranoside for 8 min at 37°C. Protein A-Sepharose was then added for 1 h at 4°C. Immunoprecipitations were washed twice with wash buffer [20 mmol/L Tris (pH 8.0), 1% NP40, 0.5% deoxycholate, 2 mmol/L EDTA, 2 mmol/L EGTA], lysates were washed twice with wash buffer [20 mmol/L Tris (pH 8.0), 1% NP40, 0.5% deoxycholate, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% SDS] and resuspended in 2× SDS sample buffer.

**References**

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