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INTRODUCTION:

This proposal is designed to explore the premise that Notch4 signaling may regulate apoptosis in the mammary gland. The specific aims are 1) to determine if nuclear hormone receptors interact with Notch; 2) to determine the functional role of Notch and nuclear hormone receptors in tumor formation: 3) to elucidate the signaling pathways influenced by potential interactions between Notch and nuclear hormone receptors. We have made significant progress towards understanding the crosstalk between Notch and some of the more important nuclear hormone receptors in the mammary gland.

BODY:

The purpose of **Aim1** was to define potential protein/protein interactions between members of the Notch family and members of the nuclear hormone receptor family. The mammalian 2-hybrid system was used instead of the yeast 2-hybrid system to look for these interactions because we felt that the mammalian cell lines COS-1 and HC11 would represent a more physiological and relevant environment. Notch4 was obtained from Robert Callahan and was fused with the Gal4 DNA binding domain. Notch1 fused the Gal4 DNA binding domain was obtained from Diane Hayward. The progesterone receptor fused to the VP16 domain was obtained from Dean Edwards lab. The interaction between these Notch family members and the progesterone receptor (PR) was analyzed in both Cos-1 cells and the mouse mammary epithelial cell line, HC11. No interaction was detected between Notch4 or 1 and the progesterone receptor (Fig.1, notch4 data sent previously).

Aim2 sought to explore the role of Notch in tumorigenesis in the mammary gland. Vectors expressing Notch1 and Notch4 were cloned and transfected into HC11 cells, a derivative of Comma D cells, which can be induced to pseudo-differentiate as well as undergo apoptosis in vitro, and the EpH4 cell line, which can form branches and lobular structures in matrigel. The Notch expressing HC11 cell lines were induced to undergo apoptosis through growth factor withdrawal and death was monitored by Annexin FITC staining. (Fig 2) Death occurred at a slightly accelerated rate in these cells, but Notch expression was diminished significantly within the first couple hours, so it is unclear whether Notch is responsible for the enhanced death or whether the degradation of Notch is responsible for the enhanced apoptosis. Recent experiments looking at the serum withdrawal-induced death of various domain mutants of the Notch1 protein have suggested that the enhanced death that was observed was not due to loss of Notch from the cell. Notch mutants that did not have a CBF1 binding (RAM) domain and or a C-terminal transactivation domain died at a more accelerated rate. These mutants did not degrade as quickly as the full-length intracellular domain of Notch1 suggesting that it wasn't the loss of Notch that caused the enhanced growth. Interestingly, the substrate or ECM provided to the cells may be altering how Notch responds to growth factor withdrawal. Future experiments will be done to examine the role of these substrates of the death regulated by Notch1.

HC11 and EpH4 expressing Notch4 intracellular region or Notch mutants with the C-terminus deleted and/or a point mutation in the 4th ankyrin repeat were plated in soft agar for analysis of the ability to grow in anchorage independent conditions. A Notch4 mutant with the C-terminus after the ankyrin domain removed appeared the most transformed with the largest and most numerous colonies forming in soft agar (Fig.3).

Transgenic mice expressing the intracellular region of the Notch4 gene under the MMTV promoter (INT3) were obtained from Robert Callahan and Gilbert Smith. Tumors and normal glands from all stages of development were fixed and paraffin embedded for immunohistochemistry and frozen for protein or RNA studies. Estrogen receptor staining did not appear to be different between transgenic and wild type controls, but the level of progesterone receptor staining was reduced by 50% in the INT3 mice (Fig4). The tumors appeared to be estrogen receptor negative, while early hyperplastic events appeared often to be associated with estrogen receptor positive cells. Western analysis suggested an up regulation of both Notch1 and Notch4 in INT3 tumors compared to transgenic "normal glands" (Fig 5 and 6). Cell lines obtained from these tumors were established and demonstrated to be growth factor-independent.

Aim3 focused on determining the signaling pathways influenced by Notch/nuclear hormone receptor interactions. Notch1 or Notch4 were transfected with various hormone receptors and response element reporter constructs to monitor for hormone-induced activity. The effect of Notch on the hormone signaling pathway was determined by equalizing the hormone activity with another reporter designed to measure transfection efficiency. Notch had a strong and reproducible effect on the activation of the progesterone receptor. Notch4 could inhibit progesterone receptor activity by approximately 50% and Notch1 could inhibit progesterone receptor activity by approximately 90-100% in HC11 and EpH4 cells (Fig7). Notch1 also inhibited PR activity in the PR expressing cell lines created by Dr. A. Molinolo (Fig 8) ruling out the effect being due to exogenously expressed PR-B. Since no interaction was observed between Notch1 or 4 and the progesterone receptor, Notch domain mutants were analyzed for the ability to repress progesterone receptor activity. These mutants, obtained from Anthony Capobianco, contain the Notch1 receptor missing the C-terminal OPA and PEST domains or the entire C-terminal transactivation domain with or with out deletion of the N-terminal RAM (CBF1 binding) domain, and a point mutation in the 4th ankyrin repeat which inhibits transformation. These studies suggested that removal of the C-terminal transactivation domain or removal of the RAM domain caused a slight relief of repression, but removal of both of these domains caused a full relief of repression suggesting that conformation of the Notch protein was likely to be required for repression of the progesterone receptor (Fig9). However, a mutation in the 4th ankyrin repeat (M1), which inhibits transformation and activation of CBF1 by Notch, does not have any effect on the progesterone receptor activity (Fig 10). These data suggest that a particular conformation of Notch is required for repression of the progesterone receptor or that two distinct signaling pathways may be involved, but that this does not occur through the transactivation of CBF1, although this does not rule out the possible importance of relief of the repressive activity of CBF1. Previously reported data has suggested that the repression is not likely to be due to increased levels or activity of Grg1, Hes1, NFkB, Bag-1, or MAP kinase signaling. We can extend these results to rule out the sequestration of co-activators such as pCAF, CBP, Src, and Rip140 (Fig.11)

The effect of Notch on estrogen receptor is quite different from that observed with the progesterone receptor. Notch expression at low levels (either stably or transiently through the use of a weaker promoter) appears to cause a slight repression of estrogen receptor activity, but Notch expression at high levels augments estrogen receptor activity (Fig12). This augmentation appears to be ligand independent in that a small, but reproducible augmentation is detected in the absence of ligand. An estrogen receptor construct containing mutated co-factor binding sites in

the AF-2 domain was obtained from Malcolm Parker and Notch could effect these mutants in a similar manner, ruling out these co-factor binding sites in the augmentation (Fig 13). The mutational analysis of Notch indicates that the augmentation of estrogen receptor activity requires the RAM domain and the mutation in the 4th ankyrin repeat (Fig 14). This suggests that the effect on the estrogen receptor may be more closely aligned with the transforming activities of Notch, which require high-level expression of Notch and an intact 4th ankyrin repeat.

KEY RESEARCH ACCOMPLISHMENTS (3rd year):

-Interactions between both Notch1 and Notch4 and the PR receptor were tested in cells, which would have the appropriate co-factors and no significant interaction was observed.
-Domains of Notch1 necessary for modulation of PR and ER were identified.
-The effect of Notch on ER and PR activity in human transformed and immortal mammary epithelial cell lines were analyzed.
-Levels of PR and ER expression were analyzed in normal and tumorigenic tissues from wild type and INT3 transgenic mice.

REPORTABLE OUTCOMES:

-Abstract presented at the DOD BCRP meeting in Atlanta, Georgia in June 2000 entitled "The Effects of Notch on the Transcriptional Activity of the Steroid/Thyroid Hormone Receptor Superfamily" SW Smith, R Lawlor, DJ Jerry, BA Osborne.

-Presentation in the University of Massachusetts, Molecular and Cellular Biology Colloquia, November 2000.

-Development of INT3 mammary epithelial cell lines
-Development of stable Notch1 and Notch4 expressing cell lines
-Development of inducible Notch1 expressing cell line
-Development of tissue collection from different developmental stages and degrees of tumorigenesis from INT3 mice.

CONCLUSIONS:

Our results suggest that Notch can modulate transcriptional activities ER, PR, GR, and RAR in mammary epithelial cells. The repression of PR activity does not appear to be through direct interaction with Notch, such as is observed with Nur77, but the RAM domain and the C-terminal transactivation domain play an important role in this activity. The domains of Notch and the dependence on level of expression required for the augmentation of the estrogen receptor do appear to correlate more closely with the requirements for transformation by Notch. While the tumors appear to estrogen receptor negative, early hyperplastic events appear to correlate with higher estrogen receptor expression. Further studies will be needed to establish the requirement for the estrogen receptor or estrogen in Notch mediated tumorigenesis of the mammary gland. The role of Notch–mediated repression of PR may be more subtle or additive to the effect on tumorigenesis. Future studies should include a closer analysis of the effect of Notch on PR and ER regulated genes *in vivo*.

The effect of Notch on apoptosis in the mammary gland is more difficult to review because the mammary epithelium in the INT3 mice fail to differentiate into lobules and secrete

milk, so are not put in the position to experience the apoptosis observed upon weaning in wild type mice. The is a slight apoptotic death that occurs during the normal estrous cycle and that has not been measured in these studies will be an important part of future studies.

The reason this research has been important is because the upregulation or activation of Notch strongly correlates with invasive human mammary carcinogenesis. The more we understand the effects of Notch expression and the pathways by which Notch exerts its effects, the better we will be able to devise strategies to inhibit its activities.

Figure 1. **Mammalian 2-hybrid assay demonstrating that Notch1 and PR-B do not interact.** Cos-1 cells were transfected by Qiagen's Superfect reagent with a Notch1-GAL4DBD construct, and VP16-PR-B construct and/or with vector alone in the presence of a GAL4 RE-CAT contruct and a beta-galactosidase construct for transfection efficiency. Empty vectors together were the negative control and p53 and large T plasmids were used as a positive control. Notch1 does have some endogenous activity normally, but the presence of PR-B does not increase the activity.

Figure 2. **Notch1 enhances death during serum deprivation**. Cells stably expressing NIC or vector alone were washed with PBS and placed in normal growth media or serum free media for 48 hours. The cells were stained with annexin V and analyzed by FACS analysis.

Figure 3. **Loss of the C-terminal domain of Int3 enhances anchorage independent growth**. EpH4 cells were stably transfected with vector alone or vector driving expression of Int3 (Notch4 intracellular domain), Int3 missing the entire C-terminus after the ankyrin repeats, or Int3 missing the entire C-terminus and with a mutated conserved alanine in the fourth repeat. Cells were plated in soft agar and grown for two weeks.

Figure 4.**The expression of PR is lower in the mammary glands of Int3 transgenic mice**. Glands from wildtype FVB parous mice or transgenic FVB parous mice were removed, fixed and embedded in paraffin. Sections were stained with an antibody to the progesterone receptor. While levels of PR looked more or less normal in ends of the ducts, the parts of the ducts closest to the nipple had little if any PR staining.

Figure 5. **Notch 1 and Notch4 expression increases in tumors from the Int3 transgenic mice.** Normal 4th inguinal glands from FVB and FVB Int3 and a tumor from the contralateral gland of the same Int3 mouse were quick frozen and lysed in protein extraction buffer. 50 ug of protein was run on a 6% SDS PAGE gel. The proteins were transferred to nitrocellulose and probed with antibodies to Notch1, Notch4, and GAPDH.

Figure 6. **Notch1 and ErbB2 levels increase in tumors from Int3 transgenic mice**. A normal gland was compared to 4 separate tumors taken from Int3 transgenic mice. Protein was extracted and run on a 6% gel. The transferred proteins were probed with antibodies to Notch1 and ErbB2 as described above.

Figure 7. **The intracellular form of Notch 1 (pCDNA NIC) represses progesterone transactivation.** NIC or vector alone were transiently transfected into HC11 cells with PR-B, PRE-Luc, and the renilla luciferase gene for transfection efficiency. Tk-Luc was used as a control. The synthetic progestin, R5020 (10nM), was given or not to the cells and the lysates were prepared 21 hours later. Promega's Dual luciferase assay system was used to monitor the luciferase activity.

Figure 8. **Notch can repress endogenous PR activity.** Mouse mammary tumor cell lines, which endogenously express PR were obtained from A. Molinolo in Argentina. NIC (N) or vector control (C) were transfected into the cells with PRE-Luc and PRL-CMV. 10nM R5020 was given to transfection and the luciferase activity was measured 24 hours later.

Figure 9. **The RAM domain and the C-terminal region are required for PR repression.** Notch1 mutants obtained from A. Capobianco were expressed in EpH4 cells in the presence of PR and PRE-Luc. R5020 was given to the cells and lysates were prepared and analyzed 21 hours later.

Figure 10. **CBF1 activity does not correlate with repression of PR by NIC.** The NIC mutants were transfected in to EpH4 cells in the presence of a CBF1 reporter assay obtained from Sophie Jarriault. Lysate were prepared and analyzed 24 hours later. Only the M1 mutation and the loss of the entire C-terminus affected CBF1 activity, which did not correlate with the relief of repression observed in Fig.9.

Figure 11. **Over expression of certain general co-activators does not relieve the PR repression observed with the expression of NIC.** CBP, PCAF, SRC, RIP140, and pCDNA were over expressed in the presence of NIC or vector alone and PRE-luc. None of them relieved repression suggesting that Notch doesn't act by sequestering or inhibiting these repressors.

Figure 12. **Increasing amounts of Int3 (Notch4) augment the activity of the estrogen receptor in the presence and absence of ligand.** 0, 1,2,4 ug of Int3 were transfected into HC11 cells in the presence of 4,3,2,or 0ug of pCDNA, respectively, and ER, ERE-luc, and PRL-CMV. 1 nM of estradiol was given to half the transfectants and lysates were prepared and analyzed 24 hours later.

Figure 13. **Notch augmentation of the estrogen receptor does not involve the co-activator binding sites.** Notch under the control of a CMV or an SV-40 promoter was transfected with vector alone, wild type ER, or ER mutated at the binding sites for Src and Rip140, and ERE-Luc. 1nM estradiol was presented to all the cells and 24 hours later the lysates were prepared for luciferase assays.

Figure 14. **The RAM domain and the conserved alanines in the 4th ankyrin repeat are required for augmentation of the estrogen receptor.** NIC mutants missing the C-terminal transactivation domain (-2202), missing the RAM and C-TAD (-R-2202), with alanine mutations (M1) were co-transfected with ER, ERE-luc, and PRL-CMVinto HC11 cells. 1nM estradiol was added and the lysates were prepares for luciferase assays 24 hours later.

Mammalian 2-Hybrid Analysis With Notch1 and PR-B

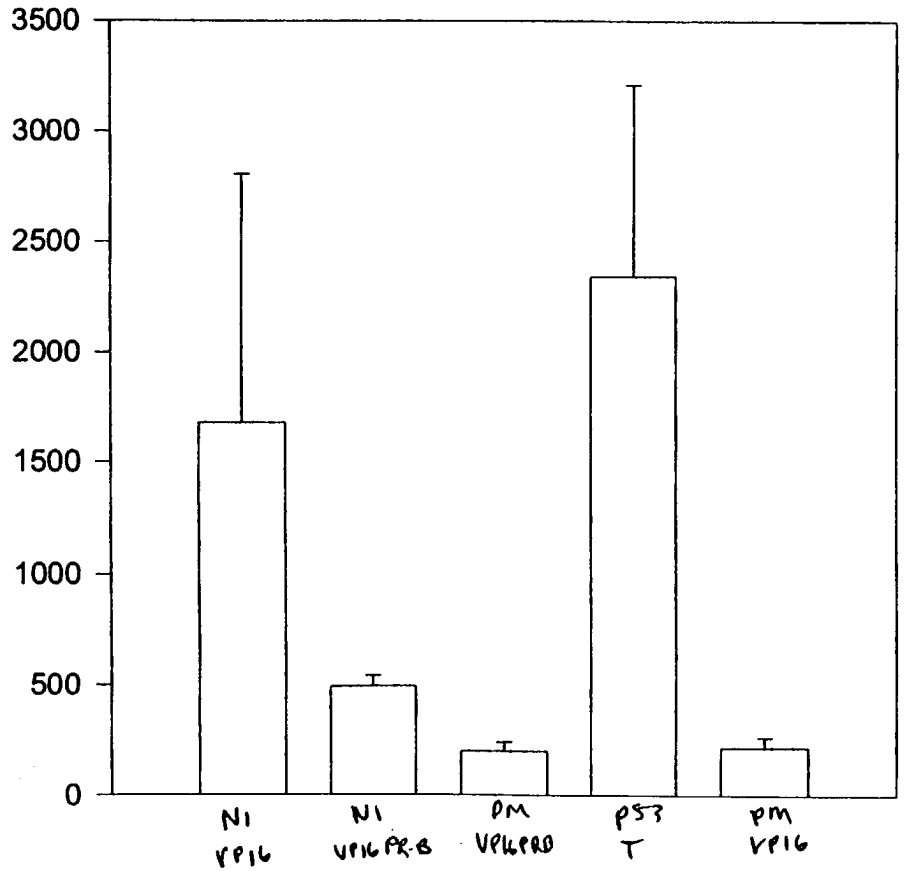


Figure 1

The Effect of Notch 1 Expression on Serum Withdrawl-Induced Apoptosis of HC11 Cells

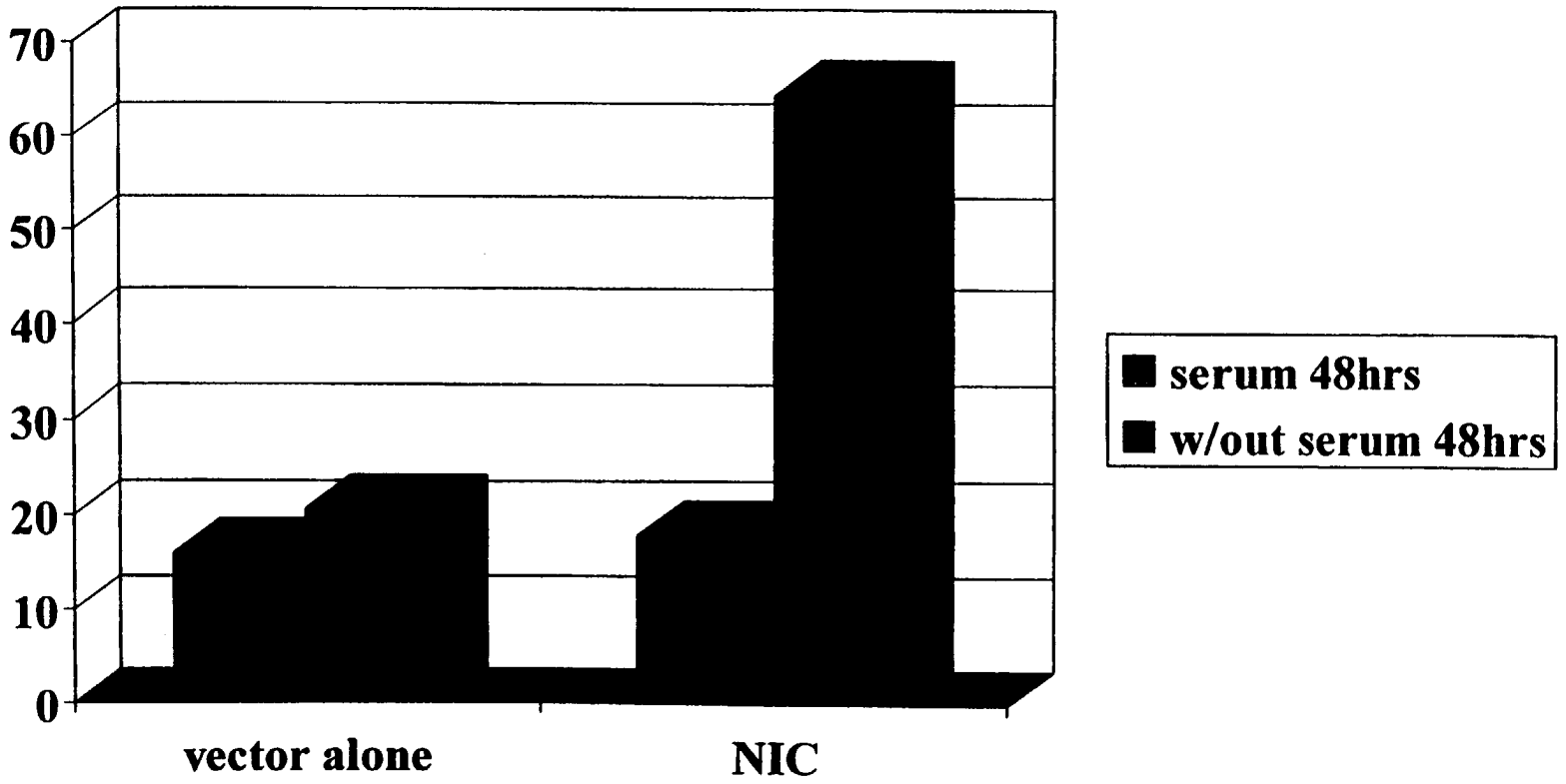


Figure 2

Figure 3

Quantity of PR expression

- Parous FVB: 31.3% of 1000, positive for PR
- FVB 3W/Int-3: 16.3% of 1000, positive for PR

Figure 4

Notch1 and 4 expression in wild type versus INT3 transgenic mammary glands

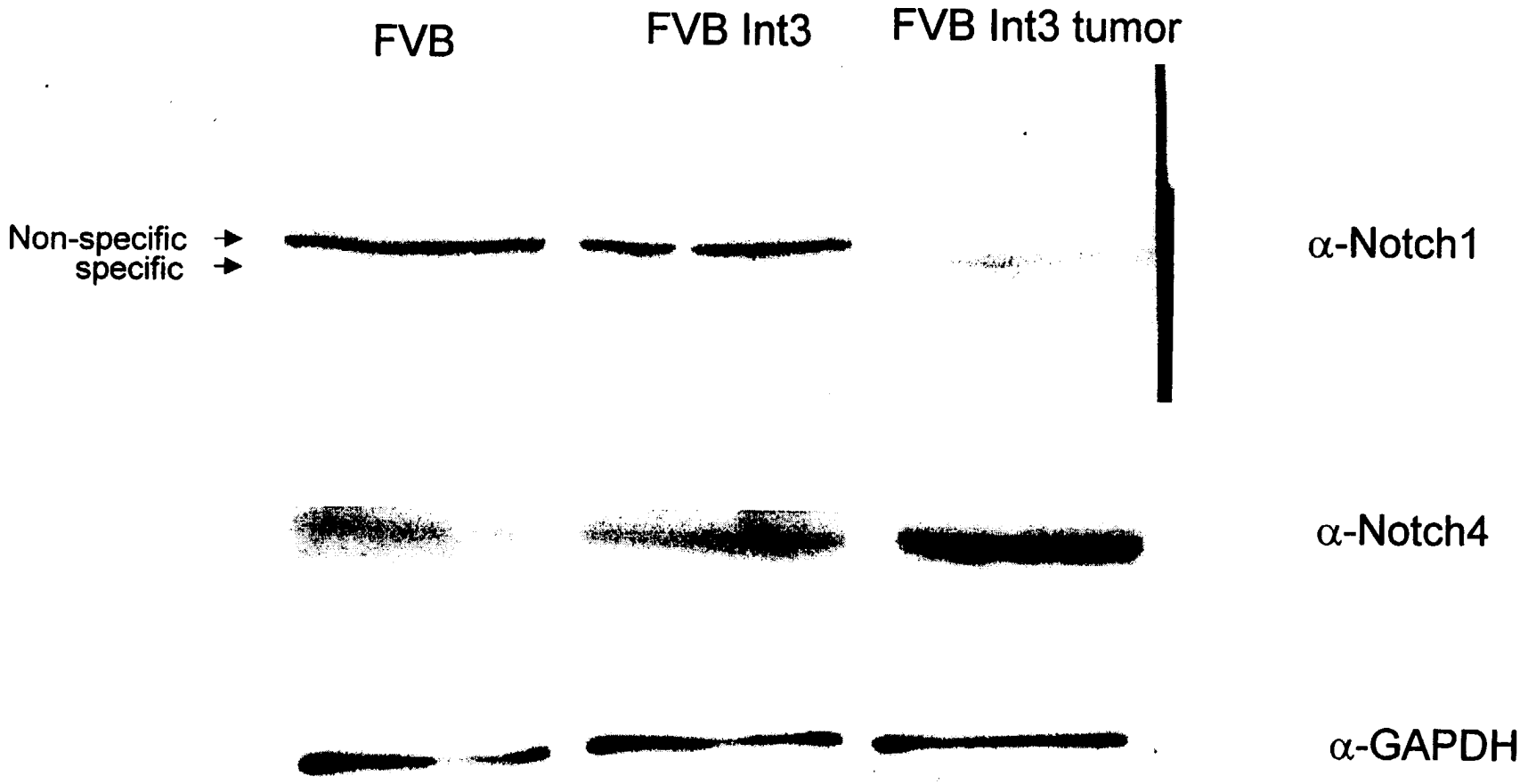
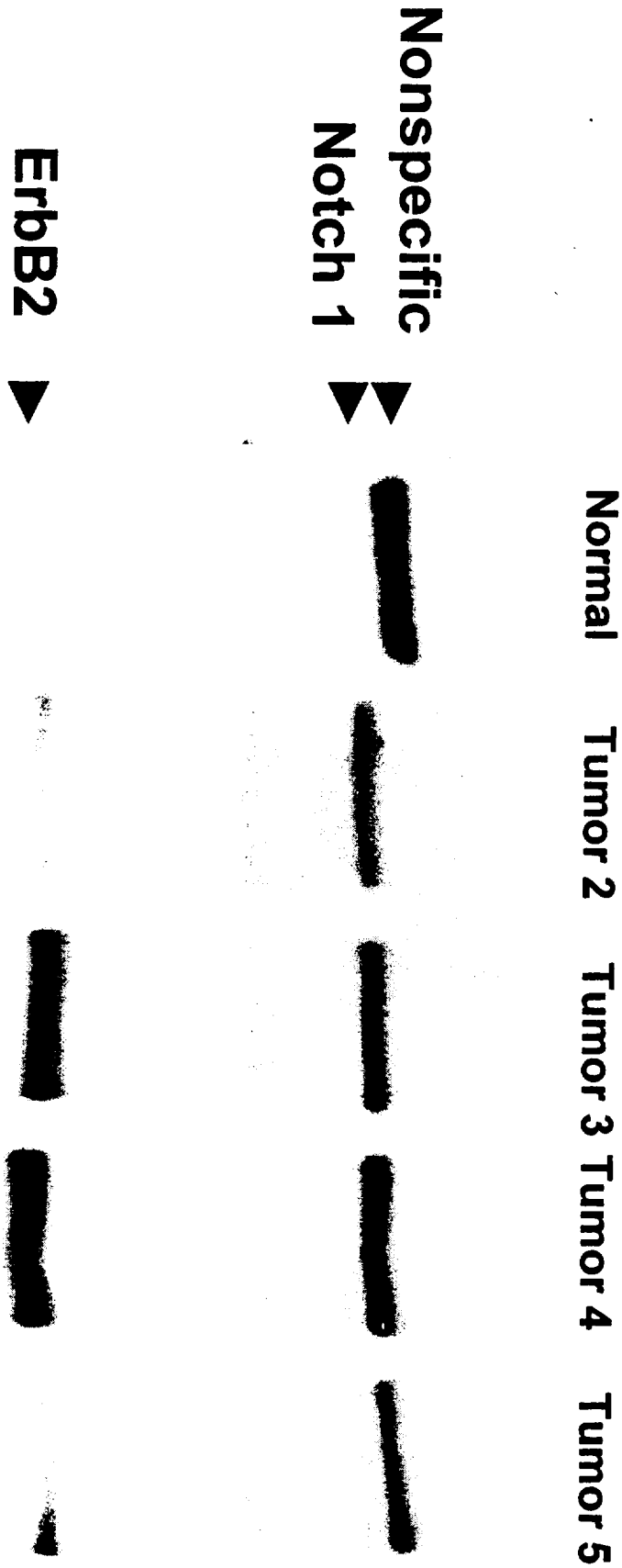


Figure 5

Figure 6


Normal
Tumor 2
Tumor 3
Tumor 4
Tumor 5
Nonspecific
Notch 1
ErbB2

The Effect of Notch1 on PR Transactivation

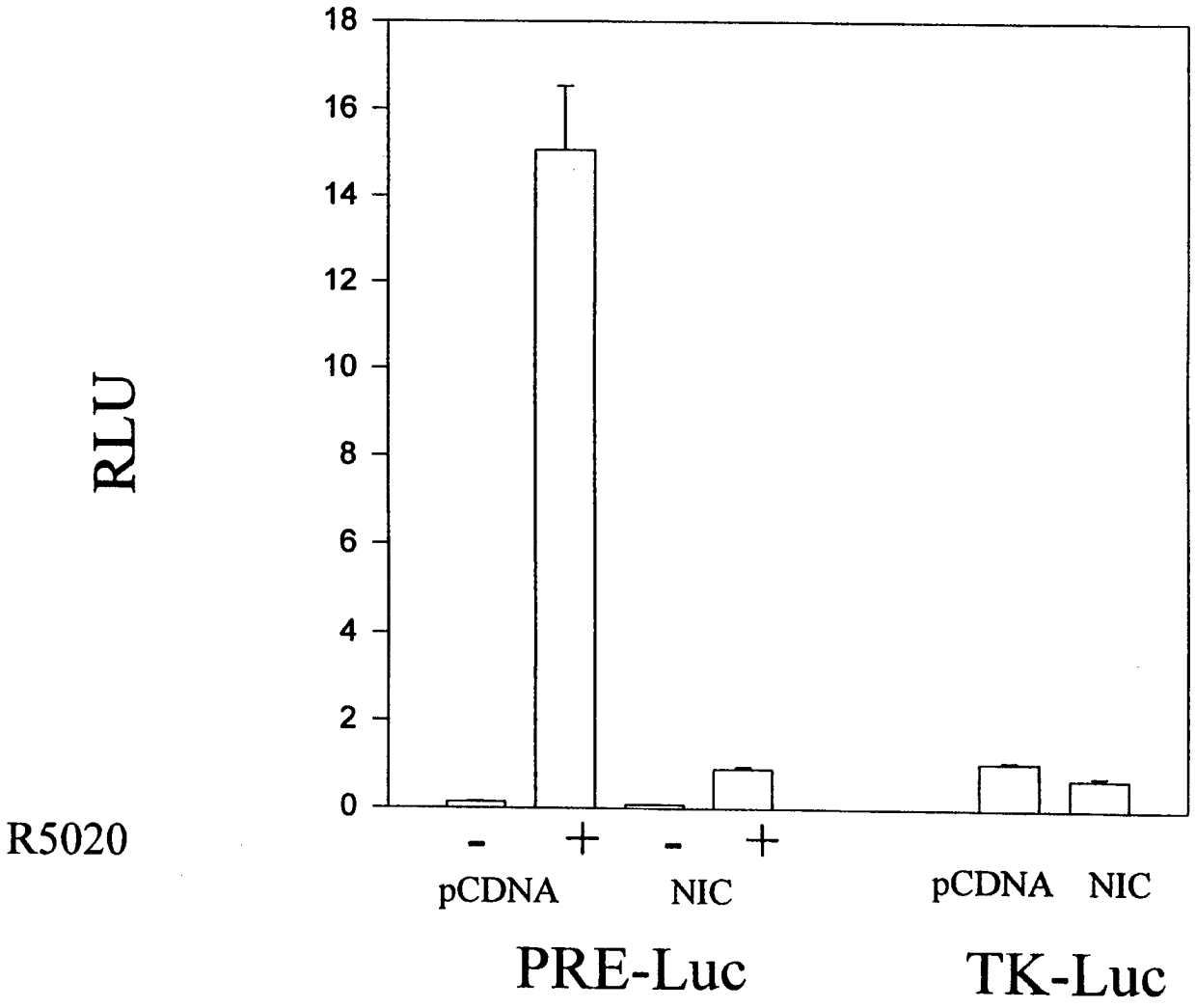
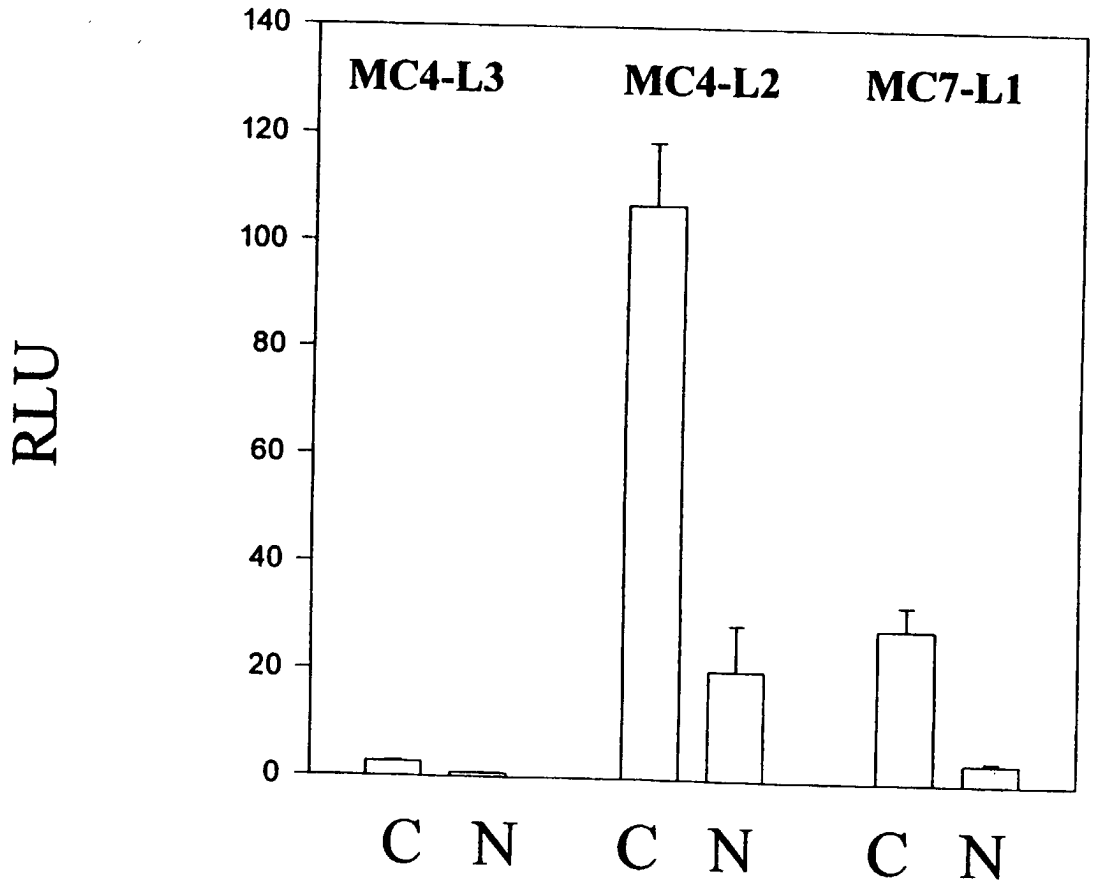


Figure 7

Endogenous PR Transactivation in Human Lines Transfected with Notch1



PRE-TK-Luc

Figure 8

Figure 9

The Effect of Notch1 Mutants on CBF1 Activity in EpH4 Cells

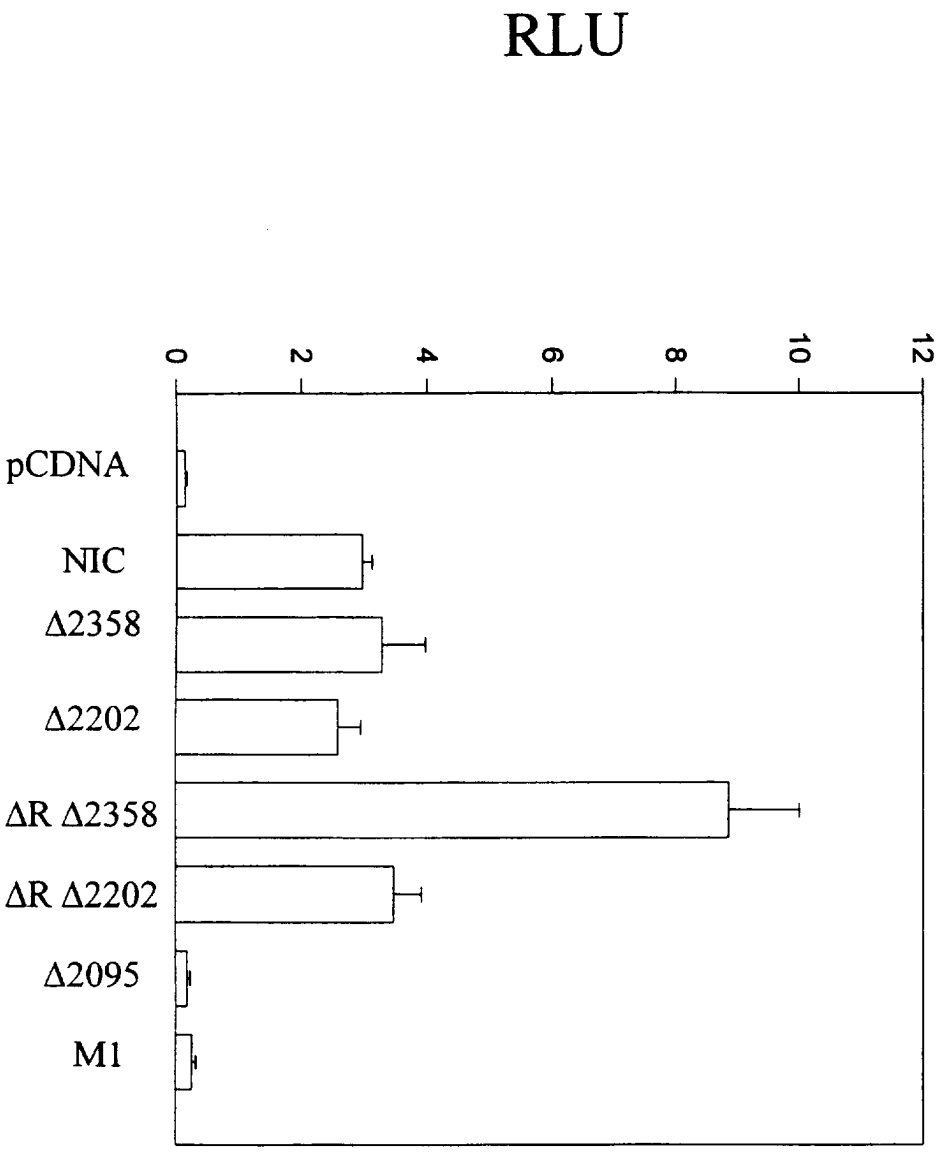
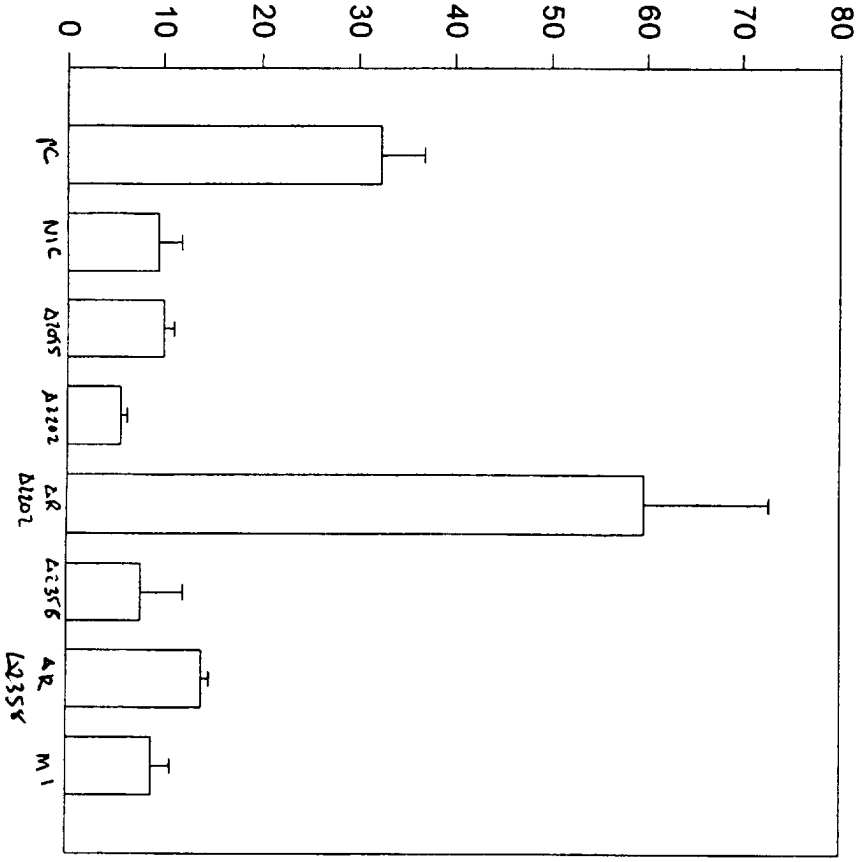


Figure 10

The Effect of Notch1 Mutants on PR Transactivation In EpH4 cells



The Effect of Co-Activators on Notch induced Repression of PR Transactivation

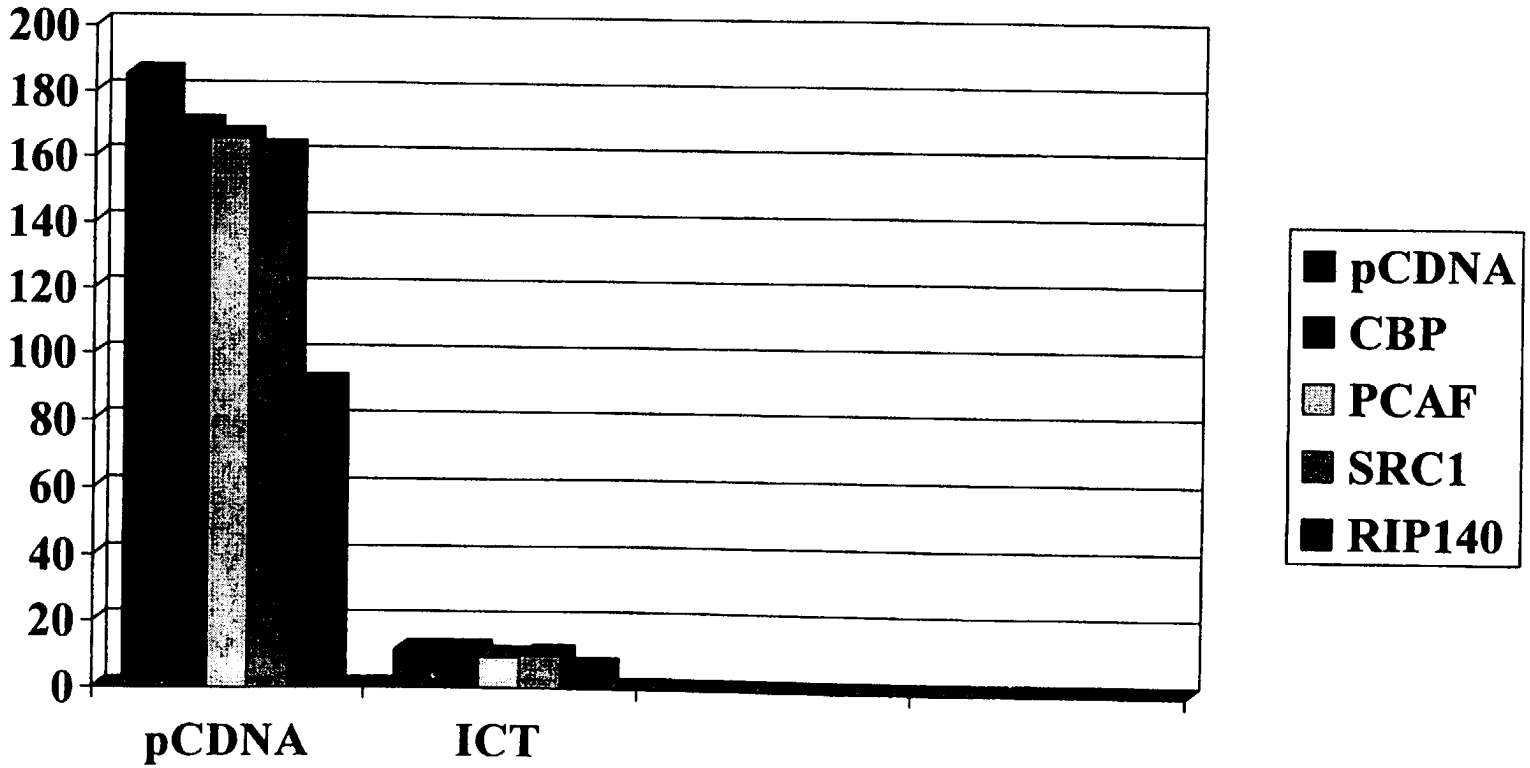
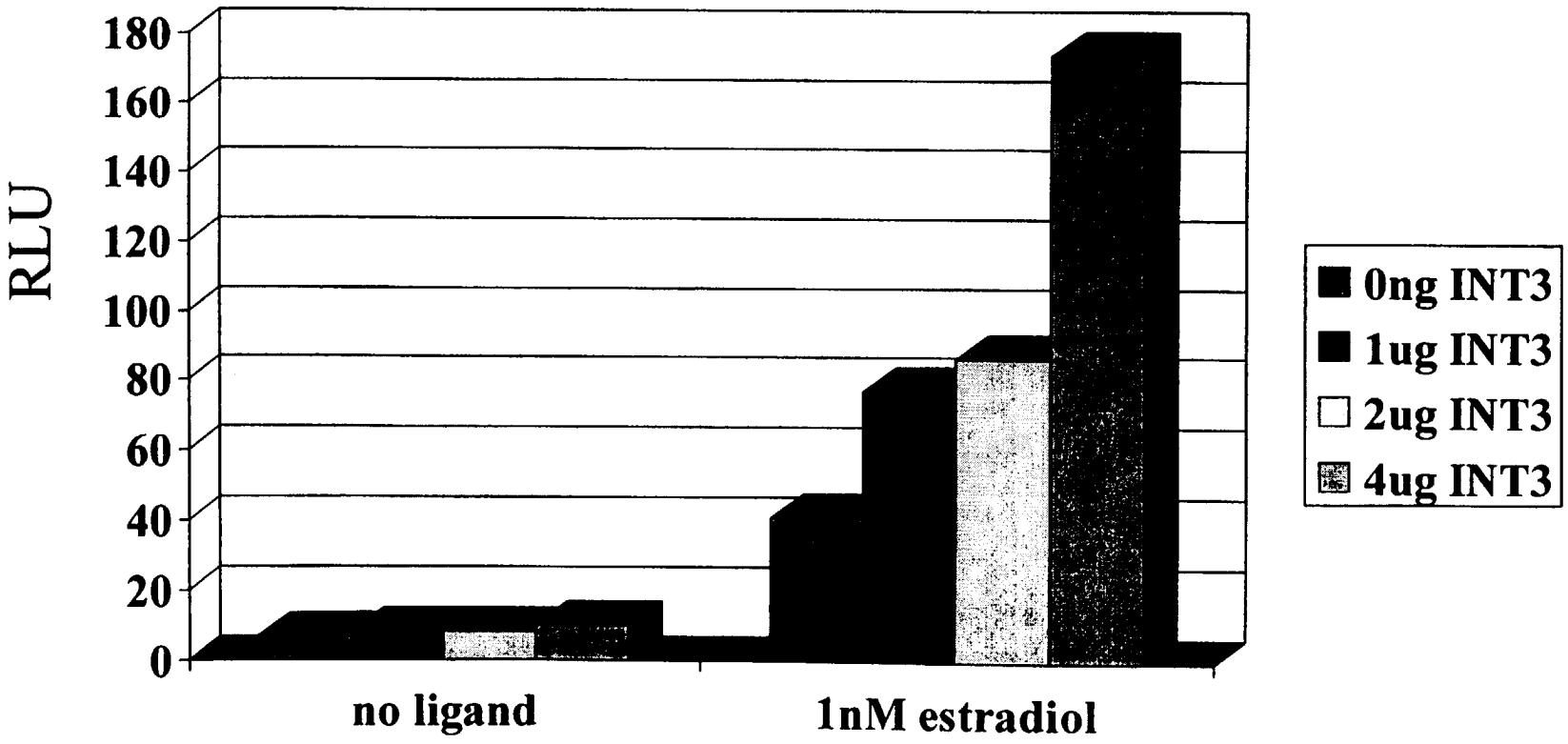


Figure 11

The Effect of Increasing Amounts of INT3 on ER Transactivation



ERE-Luc

Figure 12

The Effect of Notch4(INT3) on ER AF-2 Mutants in HC11

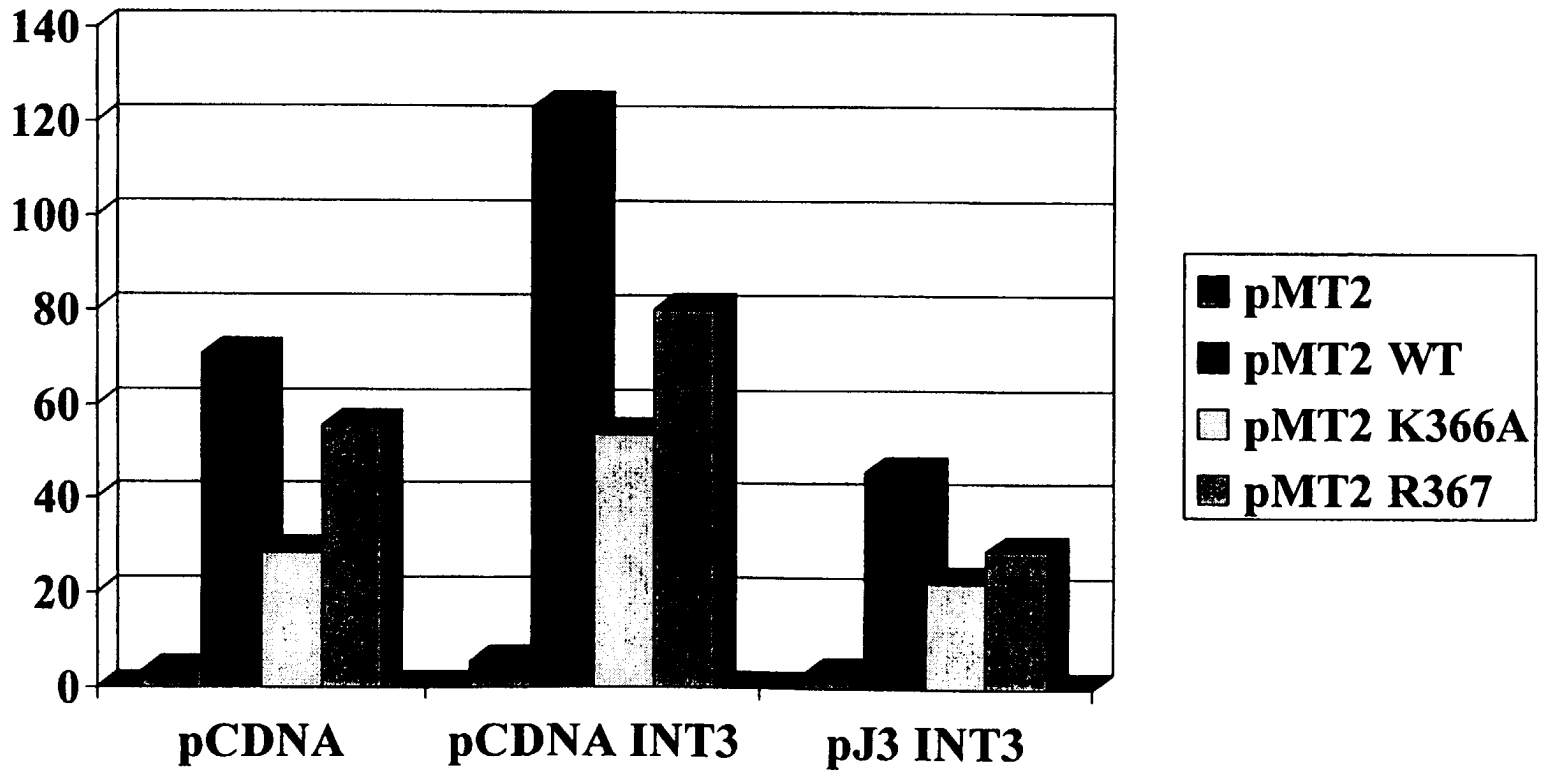


Figure 13

The Effect of Notch1 and 4 Mutants on ER Activity in Eph4 cells

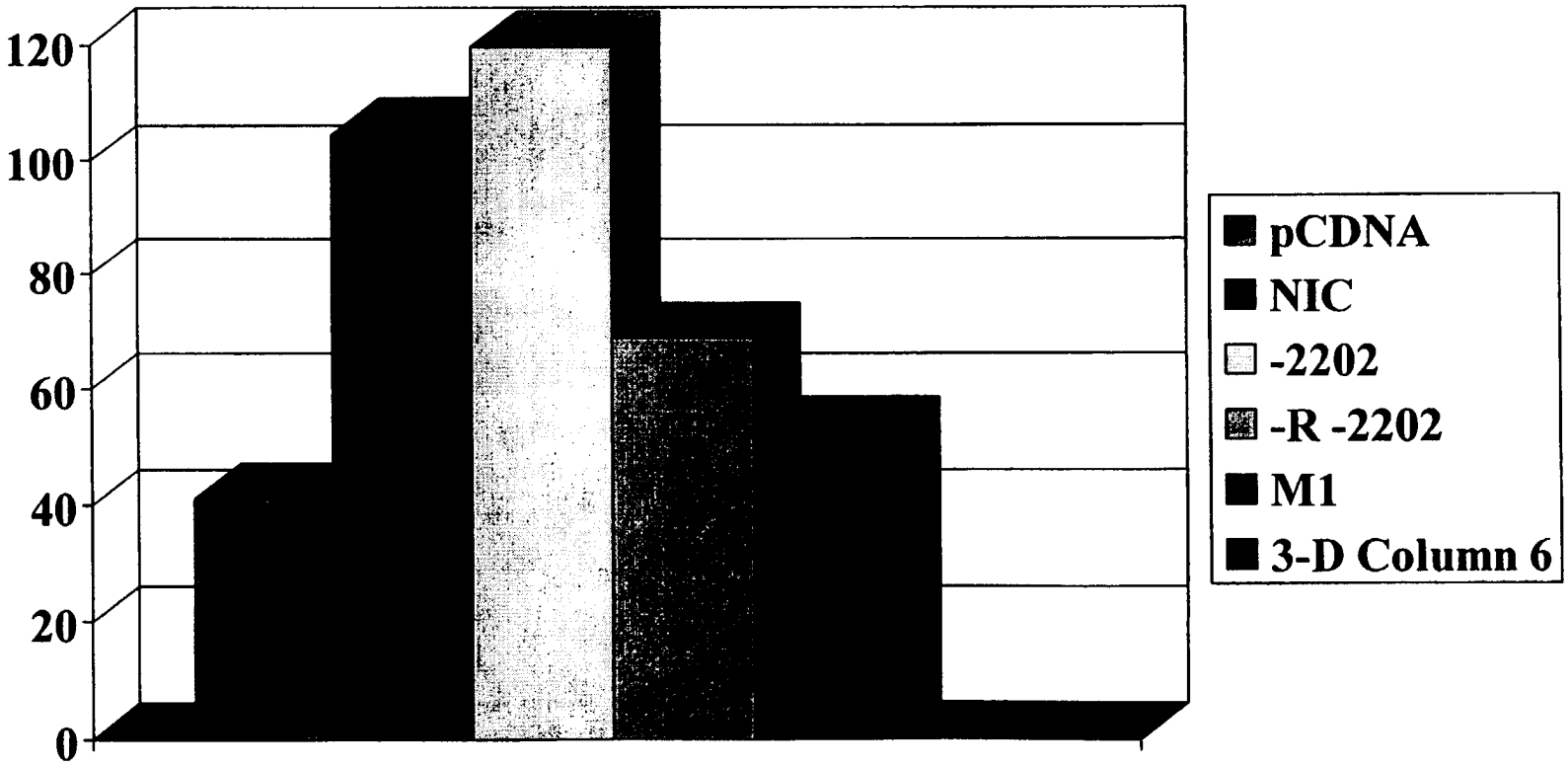


Figure 14