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Notch 1 Mediated Inhibition of Nur77-induced apoptosis: Implications for T-cell Leukemia

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NOTCH 1 MEDIATED INHIBITION OF NUR77-INDUCED APOPTOSIS: IMPLICATIONS FOR T-CELL LEUKEMIA

A Dissertation Presented

by

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ABSTRACT

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IMPLICATIONS FOR T-CELL LEUKEMIA

MAY 2010

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It is widely accepted that activating mutations of genes encoding the Notch family of transmembrane receptors, specifically Notch1, are associated with oncogenic transformation. Previous data from our lab has shown that an active form of Notch1 (Nic) provides a protective effect against apoptosis in D011.10 T cells, and that this effect may be attributed to Nic binding the pro-apoptotic protein Nur77. Nur77 is an immediate early gene that is upregulated during negative selection of thymocytes and activation-induced apoptosis in D011.10 T cells. Nur77 upregulation is tightly regulated and requires MEF2D, NFAT, and the co-activator, p300, to effectively respond to apoptotic stimuli. In this report we show that Nic has the ability to interfere with the induction of transcription of Nur77, and that this interference is directly related to the inability of p300 to bind the Nur77 promoter in the presence of Nic. We also show that blocking Notch activation through gamma secretase-inhibitors or siRNA directed against Notch1 in T cell acute lymphoblastic leukemia (T-ALL) cell lines restores Nur77 upregulation in response

stimuli. These observations support a model in which during thymocytes negative selection activating mutations of Notch1 inhibit the upregulation of a crucial pro-apoptotic molecule.

Studies to determine the mechanism by which Nur77 induces apoptosis have indentified a unique translocation of Nur77 from the nucleus to the cytosol. It has been determined that once in the cytosol Nur77 interacts with members of the Bcl-2 family of proteins at the mitochondrial membrane. This interaction induces a conformational change of Bcl-2 so that is becomes pro-apoptotic instead of protective. Of similar interest is the role that Nur77 itself plays during the induction of activation-induced apoptosis which may be independent of Bcl-2 conformational change. In an effort to describe possible functions of Nur77, DO11.10 cells that have Nur77 under a tet-inducible promoter were observed for changes IP3R. Initial results from our lab suggest that Nur77 alone has the ability to induce cell death in DO11.10 cells using this tet-inducible system. Interestingly we have been able to identify distinct changes in IP3R isoforms during stimulation induced apoptosis and Nur77-dependent apoptosis. Current experiments are focused on a mechanism beyond the known function of the Nur77/Bcl-2 interaction; that Nur77 may also be acting as a physical barrier between the known anti-apoptotic interaction of IP3R and Bcl-2, leading to sustained calcium flux.

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Chapter I

GENERAL INTRODUCTION

Introduction

The orphan nuclear receptor Nur77

Nur77, which is also known as TR3, NGFI-B, and NR4A1, is an immediate early response gene that was originally identified in PC12 cells stimulated with nerve growth factor (1,2,3). Upregulation of Nur77 was also seen to take place in thymocytes, certain cancers, and a variety of other cells in response to numerous stimuli (EGF, PMA, and TPA) (4-8). In most cases Nur77 was recognized as a factor involved in cell growth and proliferation. Research done in 1994 in our lab and in other independent labs, showed that Nur77 is a pro-apoptotic molecule in thymocytes and various different cancer cell lines (4,6,9,10).

Nur77 is recognized by its structure as a nuclear receptor and shares similarities with a large class of receptors that includes other Nur family members, steroid receptors, Vitamin D receptor, and retinoid receptor. Nur77, along with the other Nur family receptors Nor1 and Nurr1, are known as “orphan” nuclear receptors due to the lack of a known activating ligand (11). Recently it has been shown particularly in the field of cancer research, that the pro-apoptotic activity of Nur77 can by stimulated using small molecules (sodium butyrate, some phenyl methane’s) (12,13). Members of the Nur family of orphan receptors can be

found in multiple tissues including the thymus, muscle, lung, liver, testis, ventral prostate, as well as the adrenal, thyroid and pituitary glands (14).

Nur77 specific structure

As mentioned previously, Nur77 is categorized as a nuclear receptor by its characteristic structure. Like other similar nuclear receptors, Nur77 is composed of three specific domains, the N-terminal transactivation domain, a DNA binding domain that is composed of two zinc-finger motifs and a nuclear localization sequence (NLS), and a C-terminal ligand binding domain which binds the receptors specific ligands and initiates its transcriptional or transactivational activity (figure 1.1) (15-20). As expected, Nur77 shares close homology with the other members of Nur family of receptors. It has been shown Nur77 has <90% homology with Nor1 and Nurr1 in the DNA binding domain (17). The Nur77 family shows more divergent sequence homology in the N and C terminal domains, the N terminal domain shows only 27% homology between Nur77 and Nurr1 and 21% between Nur77 and Nor1. It is widely accepted that differentiation between the specific family members can be defined by difference in transactivation domains (14).

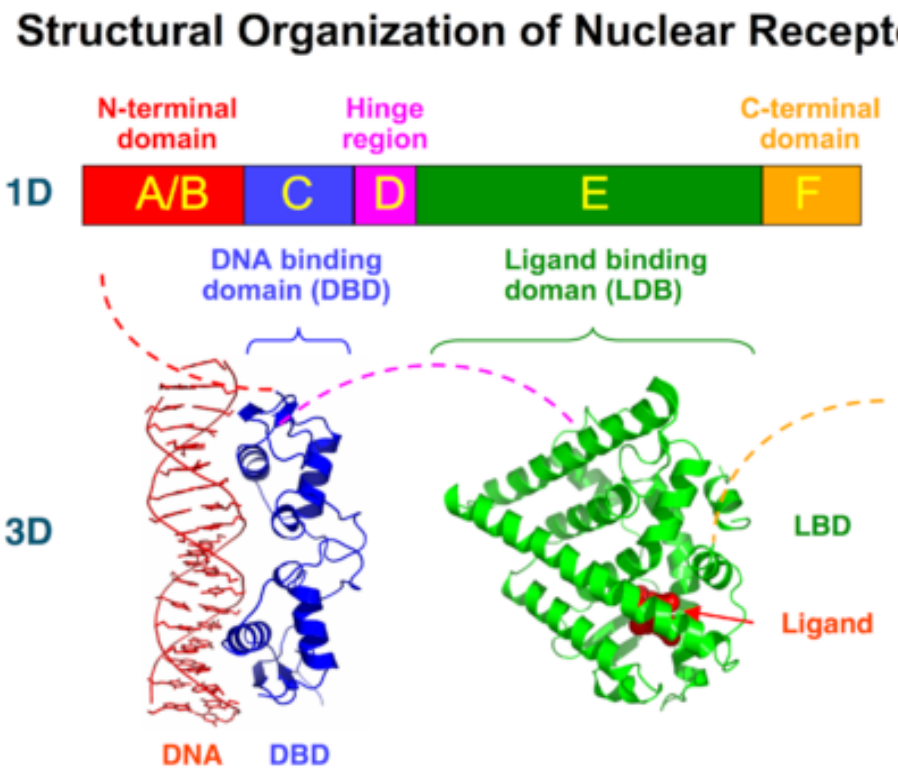


Figure 1.1: Structural organization of nuclear receptors

The 1-Dimentional make up of nuclear receptors provides information about the characteristic domains that make up nuclear receptors including Nur77. Nuclear receptors have an N-terminal transactivation domain (TAD) or A/B domain followed by a DNA binding domain (DBD) or C region. Normally there is a flexible linker or D region which connects to the E region or ligand binding domain (LBD) and, lastly, the N-terminus or F region. Shown in the 3-Dimentional structure is how the four major domains arrange themselves when the nuclear receptor is bound to DNA (21).

Nur77 as a transcription factor

Nur77 can function in the nucleus as a transcription factor by its association with specific DNA-binding response elements. Nur77 can bind as a monomer to Nur77 response elements (NBREs), which have the consensus sequence (AAAGGTCA), and is similar to the estrogen response element. Nur77 can also bind as a homodimer to Nur response elements (NurRE: TGATATTTX6AAATGCCA), and as a heterodimer with other nuclear receptors (22-24). Nur77 has been shown to heterodimerize with the retinoic X receptor, glucocorticoid receptor, and Coup-TF (25,26). Heterodimerization with these other partners has been shown to have various effects from gene regulation through other response element such as the Nur77/Coup-TF specific DR5 element (DR5: GGTTCACCGAAAGGTCA), to changes in sub-cellular localization, and decreased transcription at NBREs (27-29).

Though it is possible to identify the Nur77 DNA response element, very few Nur77 target genes have been identified and, as mentioned previously, Nur77 expression has a variety of physiological effects. Nur77 expression has been shown to inhibit IL-2 production in the Jurkat T-cell line (30). Over-expression of Nur77 in macrophages can activate IKK, the kinase that phosphorylates IκB thus activating the NF-κB pathway (31). Similarly studies using vascular endothelial cells have shown that Nur77 binds to the *IκB* promoter which upregulates IκB (32,33). This upregulation lead to a subsequent decrease in NFκB activity (32). NurRE and NBREs have been found upstream of genes in liver cells, cells of the

pituitary, and in specific cancers (28,31). In a model of activation-induced death Nur77 was shown to increase two specific genes NDG1 and NDG2 which were originally thought to be involved in its pro-apoptotic function (34). Studies from our lab have shown that expression of Nur77, which is constitutively exported from the nucleus, induces apoptosis. Though early studies of Nur77 pro-apoptotic function suggested Nur77 functions through transcriptional activation, recent studies demonstrate a very different role for Nur77 (35). Studies including those that use a pharmacological inhibitor of Nur77 DNA binding (FK506) show that transcriptional activation is not required for Nur77 induced apoptosis in several models of apoptosis (5, 36-39).

Not all Nur77 activity is associated with DNA binding activity

Though originally identified as a nuclear receptor, it has become clear that Nur77 has functions that are independent of nuclear localization. The idea of transcription factors or nuclear receptors leaving the nucleus and having a cytosolic function is not unique. Studies of p53 and the glucocorticoid receptor, which is functionally similar to Nur77, have been described actions independent of nuclear localization (40). Currently, many studies of Nur77 are focused on its activities that are not associated with its role as a transcription factor.

A seminal paper in this field shows that, in prostate cancer cells, Nur77 translocates from the nucleus to the cytosol where it targets the mitochondria to induce apoptosis *via* cytochrome C release (5). Later it was shown that Nur77 specifically interacts with Bcl-2 and, through an unknown mechanism changes

this protein from an anti- to a pro-apoptotic molecule (36). Through experiments using FK506, which inhibits Nur77 DNA-binding, and with DBD mutants of Nur77 that induce apoptosis, it became apparent that DNA binding was not required for the pro-apoptotic function of Nur77 (39).

Nur77 acts as a pro-apoptotic molecule in many different tissues and cell types including thymocytes, lung cancers, colon cancers, gastric cancers, ovarian cancers, and neurons (37). Nur77 is also involved in Sindbis virus-induced apoptosis in NIH 3T3 cells, following its translocation to the cytosol and this is inhibited by EBNA2, a Notch1-like molecule (41). Interestingly, it has also been shown that an anti-cancer agent, Apoptin, is a target of Nur77 and targets it to the mitochondria in MCF-7 breast cancer cells (43). Also, recently it was shown that Nur77 could translocate to the ER where it could bind to Bcl family members and participate in ER-stress induced apoptosis (44).

Microarray studies of thymi from wild-type and Nur77 knockout mice show minor differences in gene regulation, specifically two novel genes NDG1 and NDG2 (34). However it was later shown that these two genes are upregulated in an indirect manner and that Nur77 does not bind directly to either promoter (34). Current research from our lab has provided compelling evidence, using a form of Nur77 that is constitutively exported from the nucleus, that the cytosolic localization of Nur77 is sufficient to induce apoptosis in DO11.10 cells (45). Though not all Nur77 activities are independent of DNA-binding, it appears that its pro-apoptotic function is associated with migration to the cytosol.

Regulation of Nur77

Regulation of Nur77 can be divided into two major categories, transcriptional and post-translational, which includes changes in sub-cellular localization. Nur77 is an immediate early gene, which means that it is rapidly induced in response to specific stimuli. In the case of thymocytes, Nur77 is induced either through anti-CD3/anti-CD28 co-cross-linking, which mimics negative selection, or through stimulation with PMA and calcium ionophore. It is also clear through studies of Nur77-induction that Nur77 transcriptional up-regulation is calcium dependent (9). A major transcription factor known to induce Nur77 is MEF2D, and MEF2D binding sites are found in the Nur77 promoter. Nur77 also has two calcium dependent binding elements in its promoter (9). During TCR activation, calcium is released, activating calmodulin, which in turn binds to Cabin1 and other proteins that form a repressor of MEF2D (46). This results in release of Cabin1 from MEF2D. Once released from its repressor complex, MEF2D recruits HDAC p300 and and/or ERK5 and forms a transactivated complex of MEF2D leading to Nur77 transcription (46-52). Once transcribed, Nur77 can be modified, and recruits its own binding partners, which include ASC-2 and SMRT to increase target gene transcription (14). It has been shown that calcium/calmodulin-dependent protein kinase 4 is required for Nur77 downstream target gene transcription (14). Consistent with its action as an immediate early gene, Nur77 is very unstable. Studies in PC12 cells demonstrate a half-life of 30-40 minutes (53). Though it is not conclusive, degradation of Nur77 can be inhibited by the

proteosome inhibitor lactacystin, which suggests that Nur77 is rapidly induced and degraded.

Once translated, Nur77 is hyper-phosphorylated and has the potential to be the target of multiple kinases, though few have been identified (53,54). Analysis of the Nur77 amino acid structure reveals that it has multiple potential consensus modification motifs, which are concentrated in the N and C terminal regions. AKT has been reported to modify serine 350 (s350) of Nur77, which significantly decreases transcriptional activity (55-59). This modification also makes Nur77 a target for 14-3-3 that binds close Nur77 S350 and stabilizes the AKT mediated modification to prolong this decrease in transcriptional activity (60). This same site of Nur77 reportedly is modified by other kinases including PKA, and p90 RSK (56, 61-63). It is important to understand that modification of Nur77 is very context, stimuli, and cell type specific, which is why data concerning modification of Nur77 is often contradictory. Similarly, it has been shown that inhibitors of the ERK pathway that is activated during TCR activation decrease apoptosis in thymocytes and change the phosphorylation pattern of Nur77 (64). Likewise, in rat cerebellar granule neurons, it was shown that the MAPK/ERK pathway was able to affect localization of Nur77 in response to EGF, which retained it in the nucleus (65). Reports using PC12 cells show that the MAPK/ERK pathway can potentially modify Nur77 at serine 142, which induces its export from the nucleus (66). Nur family members have been reported to interact directly with ERK5/BMK, which both transactivates and modifies Nurr1 (67).

Most recently, detailed experiments from our lab have provided further evidence that RSK2 is intimately involved in the phosphorylation and pro-apoptotic program of Nur77 (45). Concomitantly experiments from another lab using ERK5 knockout and specific siRNA detailed the importance of ERK5 as the kinase involved in Nur77 modification (68). To this point studies using inhibitors of the ERK pathway (i.e. PD98059) have demonstrated its importance in Nur77 phosphorylation, but due to these inhibitors actions on ERK1/2, ERK5, and other possible kinases it has been difficult to identify specific kinases that directly phosphorylate Nur77 (54,64,69). Recent reports using dominant negative and knockout models of ERK5 have proved conflicts regaurding the importance of ERK5 phosphorylation of Nur77 (70-72). Interestingly, Snow *et al* has developed a specific MEK5-ERK5 small molecular inhibitor, which could be utilized to further determine this kinases specific importance (73).

Localization of Nur77

Nur77 has different actions depending on where it is localized within the cell. It contains a DNA-binding motif, which mediates its action as a transcription factor but, as mentioned, Nur77 can also translocate to the cytosol in response to various stimuli (36,74). Analysis of the amino acid sequence of Nur77 has identified a nuclear localization sequence (NLS) in the DBD, and also a putative nuclear export sequence (NES) in the LBD and serine-rich N-terminus (61,66). Similar analysis has also shown that Nur77 does not contain a consensus mitochondrial localization motif, even though publications have shown its ability

to localize to that area (5,36,37). It has been shown that the nuclear retention of Nur77 can be the result of AKT phosphorylation, that also inhibits apoptosis in the H460 cancer cell line. In this same study it was shown that de-phosphorylation at the AKT site and modification by JNK can induce nuclear export and apoptosis (54,74).

Nur77 localization has been shown to be altered by interactions with specific transactivation partners. It has been shown that intracellular Notch or EBNA2 elicits Nur77 nuclear retention and inhibition of Sindbis virus induced apoptosis in NIH 3T3 cells and Hela cells (41). It has been demonstrated in PC12 cells that Nur77 can dimerize with the retinoic acid receptor (RXR) and affect its compartmental localization leading to increased cytosol localization (67). It has also been shown that Nur77 translocation from the nucleus to the cytosol is a CRM-1 dependent event, and can be blocked *via* the CRM-1 inhibitor, leptomycin B (66). Nur77 binding with RXR has been documented in different studies and it is still unclear which protein plays the dominant role in the distribution of Nur77 (29,75). It is also clear that Nur77-RXR dimer formation can have different roles depending on conformation and cell type specificity (29,75) (Figure 1.2). This interaction has been shown to be both pro-apoptotic in cancer cell lines but also involved in transduction of extra cellular stimuli in neurons (29,75).

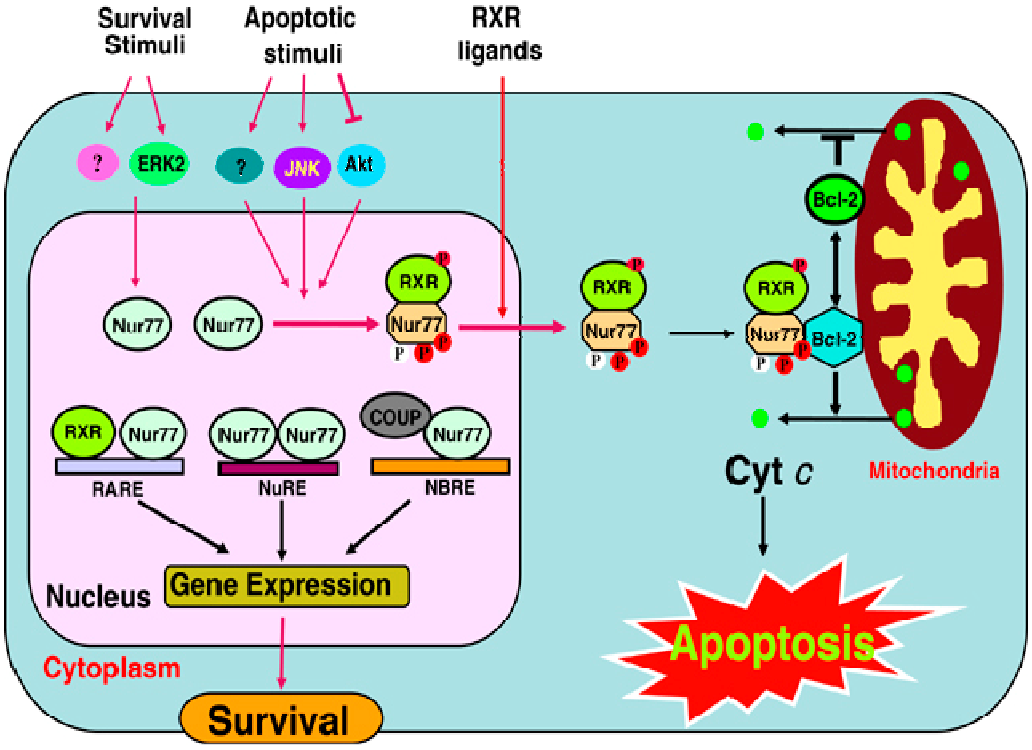


Figure 1.2: Model of possible functions of Nur77

Nur77 is an orphan nuclear receptor that is known to have functions both in the nucleus and in the cytosol. Nur77 nuclear functions include interactions with other nuclear receptors and transcription factors and gene transcription through various response elements that are influenced by the specificity of transactivation partners. Also shown is the possible importance of the cytosolic localization of Nur77 and its involvement in the pro-apoptotic program of Nur77. Additionally, Nur77 is shown interacting with RXR, however the role of RXR and its involvement in the translocation of Nur77 has yet to be definitively determined (40).

Nur77 and thymocyte negative selection

Lymphoid progenitors migrate to a specialized organ called the thymus where they are directed by various cues to mature into a single positive (SP) CD4 or CD8 T cells. The thymus has a very ordered structure; it is composed of an outer region known as the cortex and an inner region called the medulla (76). Thymocytes begin as double negative cells (DN); they go through stages DN1-4, which can be characterized by up and down regulation of specific cell surface markers. During the transition between DN3 and DN4 thymocytes go through β-selection during which they make a functional TCR β and express a putative TCRα. At this point they are referred to as double positive $CD4^+/CD8^+$ thymocytes (DP).

Thymocytes with defects in β-selection undergo death by neglect. Death by neglect makes up approximately 90% of the apoptosis that takes place in the thymus (R). this mechanism removes thymocytes that do not have the ability to interact with MHC molecules and is thought to be the result of a loss of a survival signal that is confer from the TCR/MHC interaction (77).

Recent data indicate that the glucocorticoid receptor (GR) may also play an important role during the processes of thymocyte selection, particularly death by neglect (77). The glucocorticoid receptor, as mentioned previously, is similar in structure to Nur77. Though it was originally thought that the lack of a positive stimulus was the primary reason for death by neglect interesting data is

accumulating that suggests glucocorticoid as a stimulus, instead of merely the lack of a TCR/MHC survival signal that causes apoptosis (77). Ashwell *et al* suggests there is synergism between the TCR/MHC and the GR response. In the case of thymocytes expressing a functional TCR/MHC interaction, GR could provide co-stimulation along with the specific affinity of the TCR/MHC. In case where thymocytes lack TCR interaction, GR would play a key role in the induction of apoptosis (77). Work by the same group has shown that stimulation with anti-CD3/CD28 or with glucocorticoid induces death when added individually, but when added together lead to survival (77). These results are interesting as they attempt to provide details of a process that has stymied immunologist for years. Questions still remain as to what are the contributions of GR to the process of both positive and negative selection. In the model proposed by Ashwell *et al* there is implied co-stimulatory actions from GR during the selection processes, which increases the complexity of an already dynamic system (Figure 1.3).

Thymocytes that have a functional TCR are sampled for high or low affinity to self-antigens. DP thymocytes that have a weak affinity for self-antigen will be positively selected to survive and continue to mature to SP T cells. Thymocytes that show a strong affinity for self-antigens undergo programmed cell death *via* apoptosis, also known as negative selection (Figure 1.4). How the thymocytes interpret this unique signal through essentially the same interaction remains a mystery to immunologists. Current reviews on the subject provide interesting insights that examine the affinity of the TCR by the amount of TCR that is bound

at any given time and the on-off rate of the TCR-MHC interaction as possible mechanisms (77).

It is still unclear how the same TCR can be used to transmit two extremely different signals, though it has become clear that calcium flux and kinase cascades play important roles in both processes. Studies have shown that calcium-regulated transcription factors like CABIN, HDAC7, and MEF2 play key roles in regulating downstream genes involved in negative selection (48,77). The most important downstream genes that are upregulated during the process of negative selection are thought to be members of the BH3 only family of pro-apoptotic molecules such as Bax, Bad, and Bim (48,77). The proteins of the BH3 only family are thought to induce apoptosis by inhibiting the anti-apoptotic Bcl-2 family of mitochondrial proteins, which leads to mitochondrial instability (48,77). Our labs, as well as others, have provided evidence that the immediate early gene Nur77 is intimately involved in the process of negative selection and is thought to be pro-apoptotic (4,5,77). Many groups have shown that a multitude of kinases such as p38, JNK, and the multiple MAPK pathways are important to the progression of negative and positive selection (48,77). Both negative and positive selection have been extensively studied due to implications in autoimmune disease, cancer development (48,77).

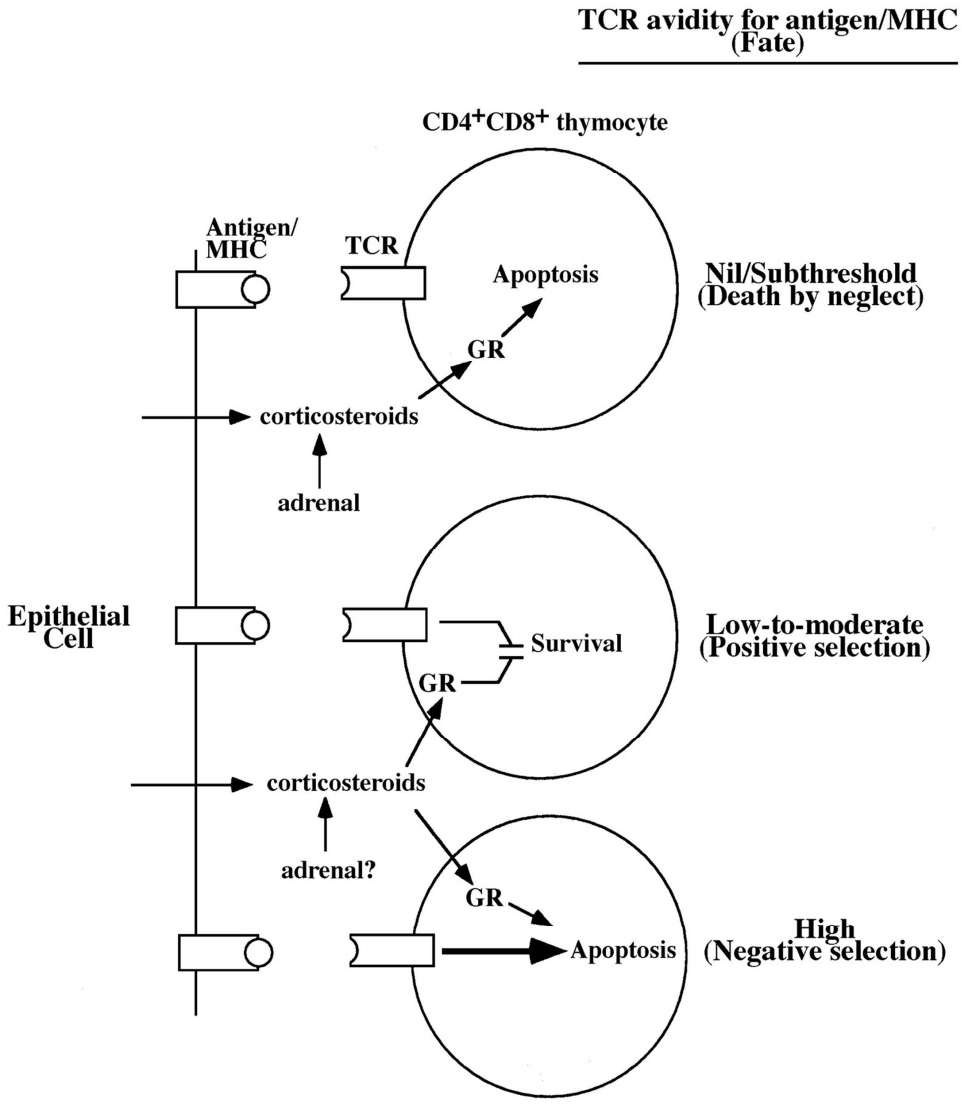


Figure 1.3: The Glucocorticoid Receptor and T-cell development

In the model proposed by Ashwell *et al*, the glucocorticoid receptor plays a vital role in the process of death by neglect. As seen in the diagram that is detailing "death by neglect" in the case of sub-optimal or no TCR interaction GR plays a vital role in this specific form of apoptosis. In instances of either positive

or negative selection, the role of GR is less clear, but they suggests a possible co-stimulatory role (77).

Experiments conducted in our lab were able to show that the immediately early gene Nur77 is upregulated during TCR stimulation-induced apoptosis in both thymocytes and T-cell hybridomas (4,9). Over-expression studies of Nur77 showed significant increases in thymocyte apoptosis (78,79). Similarly, expression of a dominant negative form of Nur77, which is made through deletion of the N-terminus, showed an inhibition of thymic negative selection (4,9). Studies using Nur77 knockout mice show little or no phenotype which is the result of functional redundancy between Nur family members specifically Nor1 (38,80). Recently, studies that have made Nur77/Nor1 double knockout mice show a severe phenotype that is highlighted by development of AML (81).

Although it is clear that Nur77 and other Nur family member are important in thymocyte negative selection, the mechanism of action is still unclear. Initially it was believed that since Nur77 is a conventional transcription factor, its pro-apoptotic functions involved downstream gene regulation (35). Early studies prematurely suggested that Nur77 was involved in fine-tuning the sensitivity of Fas/FasL interactions (34,80,82). Nur77 was also thought to upregulate another TNF like molecule CD30, but as with the Fas/FasL studies this remains controversial (83). One group has used microarrays of fetal thymi from Nur77 knockout and from wild type animals to identify two unique genes termed NDG1 and NDG2 (84). However, further studies have shown that Nur77 indirectly influences the up regulation of these genes since there are no Nur77 recognition

elements in the promoter. As was mentioned earlier, studies using FK506, which inhibits Nur77-DNA binding, do not show a decrease in apoptosis in response to TCR stimulation (39).

Also described in previous sections, work from our lab has identified RSK2 as a kinase involved in Nur77 induced translocation and pro-apoptotic programs (45). RSK2 is known to be present in DO11.10 cells, which are a thymocyte hybridoma, and in primary thymocytes as well (45). Results from our lab provide compelling evidence that Nur77 over-expression is sufficient to induce apoptosis in DO11.10 cells (45). Similarly, the pro-apoptotic phenotypes of the cells used in the study were increased when a cytosolic only form of Nur77 was used, as opposed to wild type Nur77 which showed localization to both the nuclear and cytosolic compartments (45).

For these reasons we believe that Nur77-induced apoptosis in thymocytes undergoing negative selection is independent of DNA binding and instead involves Nur77 translocation to the cytosol where it is involved in the mitochondrial-dependent apoptotic pathway *via* interaction with Bcl-2 (Figure 1.5).

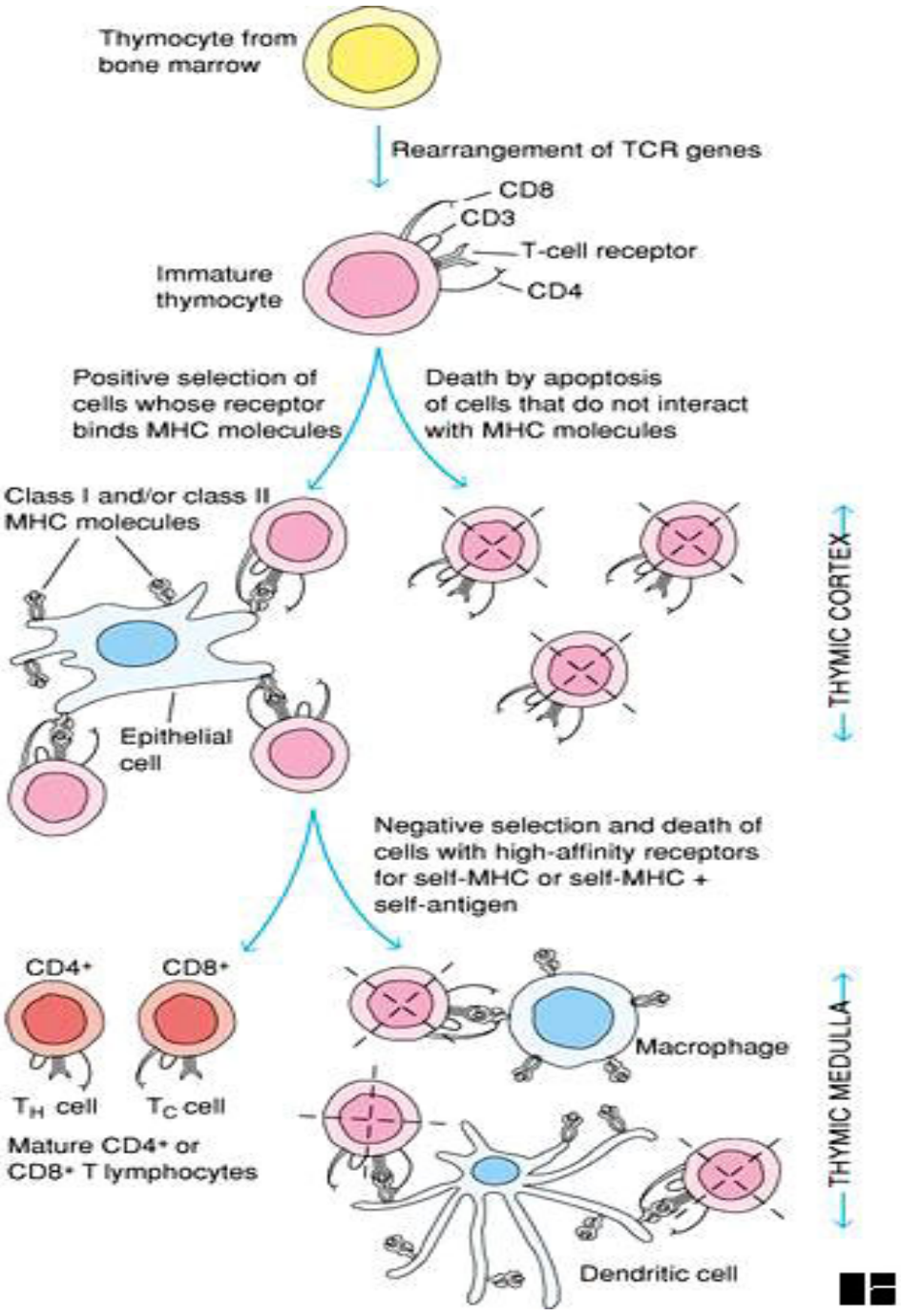


Figure 1.4: T-cell development in the thymus

Progenitors that are derived from the bone marrow migrate to the thymus where the become thymocytes. As mentioned earlier, there are characteristic markers for determining specific stages of maturation. As shown above, thymocytes undergo three major processes of selection. Thymocytes that do not interact with self-MHC at all undergo death by neglect. Cells that interact weakly with self-MHC are positively selected and allowed to survive. Cells from the same pool are negatively-selected if they interact too strongly with self-MHC or self-MHC/self-peptide complexes and undergo apoptosis (85).

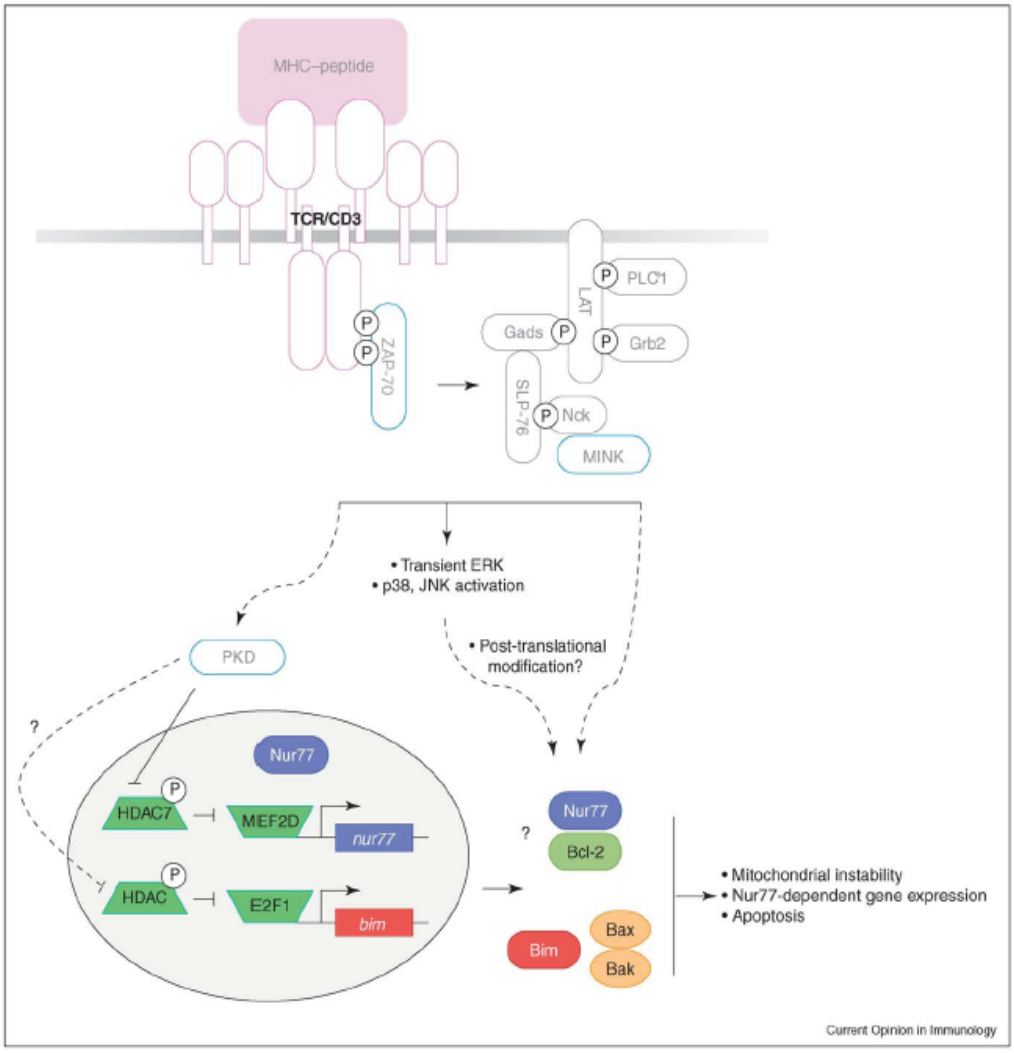


Figure 1.5: Nur77 involvement in thymocyte negative selection

As described in detail earlier, the process of negative selection involves multiple signaling pathways which include the upregulation of immediate early gene Nur77. It is hypothesized that Nur77 can affect negative selection both through the upregulation of Nur77-dependent genes, and through it's ability to be exported

from the nucleus to the cytosol and interact with Bcl-2 at the mitochondrial outer membrane (76).

The Notch family of transmembrane receptors

The gene that encodes the Notch receptor was originally characterized in *Drosophila* as a haplo-insufficiency that resulted in "notched" wings (86). Notch proteins are a large family of single pass transmembrane receptors that, in mammals, consists of 4 isoforms (Notch 1-4). Activation of Notch receptors involves cell-to-cell contact and binding of Notch cell surface ligands jagged 1 or 2 (Serrate family) or Delta 1, 3 or 4 (Delta family) (87).

The Notch protein is synthesized as a 300 kD holoreceptor that contains an extra-cellular portion, an intra-membranous portion, and an intra-cellular, membrane bound portion (88). The extra-cellular domain of Notch which contacts its cognate ligand is composed of up to 35 EGF like repeats and 3 cysteine-rich LIN-12/Notch like repeats (86). The EGF-repeats are directly involved in ligand binding, whereas the LIN-12/Notch repeats negatively regulate ligand independent cleavage (86,89,90). After interaction with cognate ligand the extra cellular portion of Notch1 is cleaved by a ADAM metalloprotease. This interaction is thought to induce a conformational change in the receptor making it accessible to the gamma-secretase complex. Liberation of the intracellular portion of Notch (Nic) renders it transcriptional active (91). Nic consists of an N-terminal RAM domain, multiple ankyrin repeats which mediate complex formation, two NLS sequences, and a PEST domain that is used to mediates Nic degradation

(92). Nic, once activated, translocates to the nucleus where it forms a complex with CBF-1, thus up-regulating downstream genes (93). Published and unpublished reports from our lab have shown that Nic also has the ability to interact with NF-κB and alter its translocation (94).

Notch is involved in numerous different cellular and developmental processes, one of which is the process of programmed cell death. Notch1 has been shown to inhibit apoptosis in mouse and human beta cells as well as other models (41,93). As mentioned before, in a study of Nur77 mediated apoptosis in NIH 3T3 cells, Nic was shown to inhibit apoptosis by sequestering of Nur77 in the nucleus (41). Not surprisingly, Notch1 has been implicated as an important factor in various cancer models. Notch1 deregulation has been shown to be a pro-survival factor in many cancers including breast cancer, colon cancer and lymphoma (94). Drug studies using gamma-secretase inhibitors, which block the cleavage and activation of Nic, have been shown to induce growth arrest and apoptosis in some cancer models (95-97). It has become widely acknowledged that some specific types of cancers are "addicted" to Notch signaling, highlighting the vital role of Notch activity in some neoplastic disease.

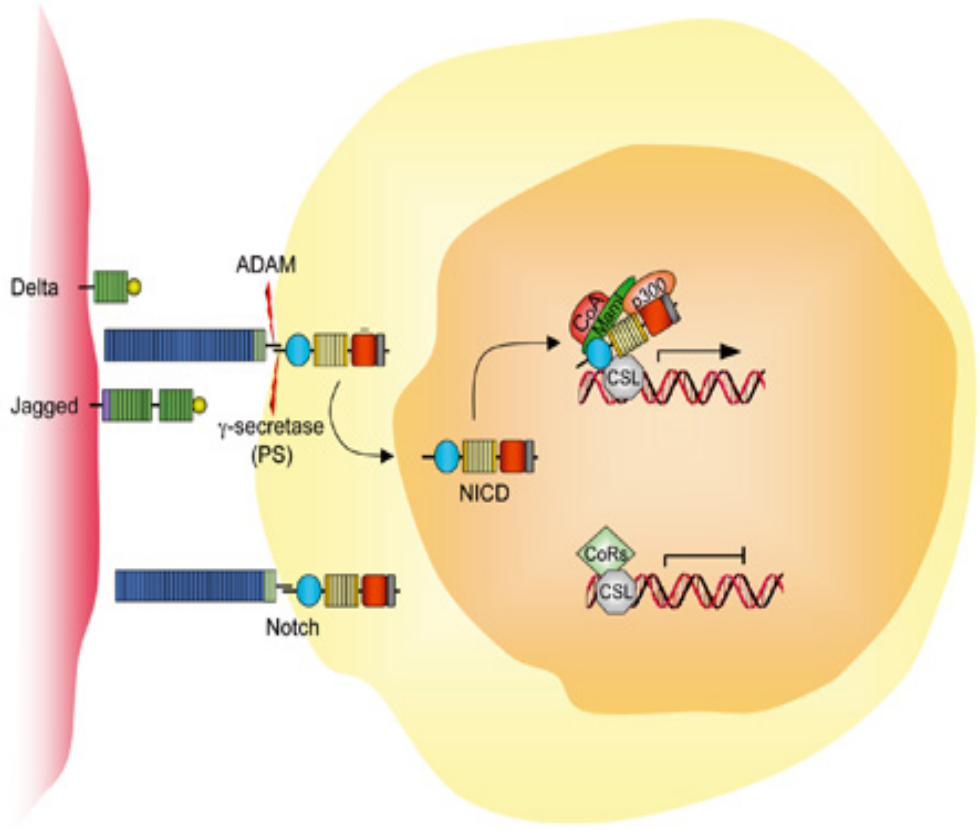


Figure 1.6: The Notch1 signaling pathway

Notch1 is a heterodimer, which consists of an extra-cellular and intra-cellular portion. Upon interaction of the extra-cellular portion of Notch1 with cognate ligand, the receptor as a whole undergoes a series of cleavage events that drive Notch1 mediated signaling. As shown, an ADAM metalloprotease cleaves the extra-cellular domain, and gamma-secretase cleaves the transmembrane-tethered intracellular portion releasing the active form of Notch1, which translocates to the nucleus and interacts with CSL and other co-activator like MAML1 and p300 (98).

Notch1 and T cell leukemia

T-cell Acute Lymphoblastic Leukemia (T-ALL) is characterized by increased numbers of immature $CD4^+/CD8^+$ double positive T-cells in the periphery. T-ALL constitutes about 15-20% of all forms of ALL cases that are diagnosed and until recent advances, normally came with a fairly grim prognosis. Notch1 involvement in T-ALL development was initially identified as a t(7:9) chromosomal translocation that leads to a truncated form of Notch1 (TAN1) in humans, but is found approximatly in 1% of T-ALL cases (95). More recent studies have shown that other mutations in Notch1 can have similar effects. Experiments by Weng *et al* used T-ALL cell lines to identify gamma-secretase inhibitor (GSI) sensitive lines and characterized specific mutations in these cell lines (99). GSI sensitivity was determined by observing increases in cell cycle arrest of the treated cell lines. Later studies also were able to determine that exposure to GSI in some of the cell lines studied induced apoptosis. The results of this study identified naturally occurring mutations in the heterodimerization domain, which lead to an auto-activating cleavage of Nic from the membrane, and truncation of the PEST domain that resulted in retention of active Nic (100) (Figure 1.7). Further studies to characterize Notch1 mutations in T-ALL showed that 56% of all the samples showed at least one possible activating mutation, and 16% had more than one mutation (100).

Activated Notch1 is considered to be the primary oncogenic molecule in the development of T-ALL. Studies in mice, also which have activated Notch1 under

the control of tetracycline-inducible promoter, show that Nic over expression leads to increased incidences of T-ALL-like disease (101). The use of these same mice, known as TOP-NOTCH mice, have been used to examine the multiple mechanisms by which Nic over-expression may contribute to T-ALL development. Nic in this mouse model has been shown to inhibit p53 activity by interfering with the mdm2-p53 interaction (101). In this same system Nic has been shown to cause upregulation of the known oncogene, c-Myc (102). A number of noted reviews describing Nic as an oncogene show more and more possible targets of Nic over-expression and targeting of multiple cellular processes (R). Early studies in our lab have shown that Nic has the ability to decrease apoptosis of the thymocyte hybridoma cell line DO11.10, showing a direct interaction with the pro-apoptotic molecule Nur77 (103). As mentioned earlier, Nic over-expression has been reported to change the localization of Nur77, which also lead to a decrease in its pro-apoptotic functions.

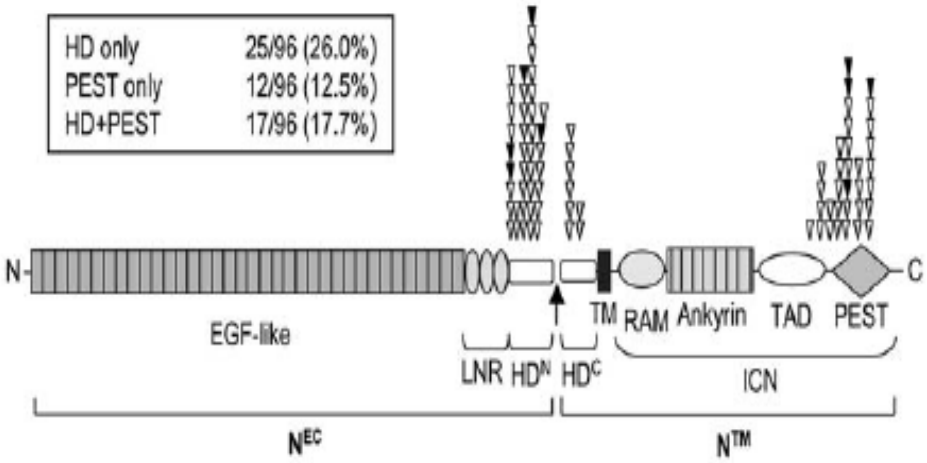


Figure 1.7: The Notch1 and T-ALL

Activating mutations in Notch1 have been found in sample from over 50% of patient samples with T-cell Acute Lymphoblastic Leukemia (T-ALL). Along with the known chromosomal mutation with leads to a truncated active form of Notch1, point mutations in Notch1 have been identified which all lead to increase active Notch1. Mutations in the Heterodimerization domain (HD), the PEST domain or both make up a large percentage of the activating mutations identified to date. Mutations in the HD domain lead to ligand-independent cleavage of Notch1, while mutations in the PEST domain are thought to lead to retention of active Notch1, by decreasing protein turnover (99).

IP3R receptor and calcium release

Inositol 1,4,5-trisphosphate receptors (IP3R) are ligand-dependent, gated, pore-forming channels that enrich the endoplasmic reticulum (ER). IP3R are very large (~1200 kD) multi-domain proteins with an N-terminal domain that is exposed to the cytosol and a C-terminal domain that forms the membrane channel (Figure 1.8). The N-terminal region constitutes the area that binds ligand and is the target for other modifications and cleavage events. IP3Rs have three different isoforms IP3R 1-3, and splice variants of all three different IP3Rs have been identified. The primary function of IP3R is to release stored calcium from the ER, in response to specific stimuli. Calcium release from the ER *via* the IP3R is involved in a diverse set of functions including muscle contraction, motility, fertilization, proliferation, calcium responsive gene regulation, and apoptosis. It is also important to point out that the IP3Rs and ER are in close contact with the mitochondria and, normally, calcium from the ER feeds into the mitochondria and helps power ATP synthesis. Normal cytosolic calcium are approximately 100nM. It has been shown that IP3Rs are sensitive to calcium changes up to approximately 500nM. Above that threshold, IP3Rs tend to be in an inhibited, closed state (104,105).

IP3R, calcium and apoptosis

Early studies in DT-40 TKO (chicken B-cell lymphoma) cells, which have all three forms of IP3R deleted show that they are resistant to apoptotic stimuli. It has

been shown that TCR- and BCR- activation induced apoptosis involves generation of IP3 and calcium release *via* ER IP3R gated stores (104,105). Other apoptotic stimuli that are known to involve calcium release are TNF alpha, Fas/FasL, ceramide, STS, cisplatin, and PMA/Ca ionophore (104,105). It is thought that conversion of IP3R from a regulator to aiding in apoptosis is due to an uncontrolled release of calcium, which overloads and, possibly, destabilizes the mitochondria (104,105). This destabilization leads to the release of cytochrome C and other pro-apoptotic factors from the mitochondria resulting in caspase activation. Studies in Jurkat cells that had decreased levels of IP3Rs showed a decrease in apoptosis and caspase activation in response to anti-CD3 cross-linking (104,105). Though it is not clear which isoform of IP3R is important in apoptosis, it was shown in cells undergoing apoptosis that IP3R-3 is highly-enriched and that anti-sense RNA to IP3R-3 could abrogate apoptosis (104,105). Similarly, it was shown that Jurkat cells that have reduced levels of IP3R-1 are deficient in response to specific apoptotic stimuli (104,105). Animal models that consist of an IP3R-1 knockout or an IP3R-2+3 double knockout show little or no phenotype, suggesting that there is some redundancy of function between the multiple isoforms (104,105).

Though it has been established by many model systems using knockout and over-expression studies that IP3Rs are involved in apoptosis, it is still unclear what leads to its change of function from a regulatory to a pro-apoptotic molecule. It has been suggested that the phosphorylation state of the IP3R N-terminal region could play a role in this transition. Studies have shown that IP3Rs can be

modified by A kinases that favors calcium release, and G kinases, which inhibit calcium release (104,105). IP3Rs are also a known target of AKT, PKC, cdc2/cyclin B1, and various MAP kinases (104,105). AKT in particular, has been studied due to its known anti-apoptotic effect *via* various pathways. Phosphorylation of IP3R by AKT was shown to be variable and not directly involved in calcium release, however, it has been hypothesized that specific kinase modification could be protective by inhibiting other protein interactions (104,105). Other studies have shown that anti-apoptotic members of the Bcl-2 family are involved in preventing apoptosis induced *via* IP3R, specifically, Bcl-2 itself and Bcl-XL. It has been well-established that along with localizing to the mitochondria, Bcl-2 family members are localized at the ER and may function in a stabilizing role (104,105). IP3R has also been shown to be a downstream target of caspase 3 and a binding partner of cytosolic cytochrome C (104,105). Cleavage of IP3R by caspase 3 results in a 215kD portion, as well as a 95kD portion that is independent of regulation essentially locked in an open, calcium-releasing state (104,105)

Thymocyte negative selection is a complex process that clearly involves the Nur family of orphan receptors, specifically, Nur77. It is also abundantly clear through past and current research that calcium flux from the endoplasmic reticulum *via* IP3R is also involved in this vital process. The goal of our research in negative selection is to further define the roles of Nur77 and IP3R during apoptosis. Our research also strives to highlight intracellular Notch disregulation and its interaction with Nur77 during cancer development in a model of T-ALL.

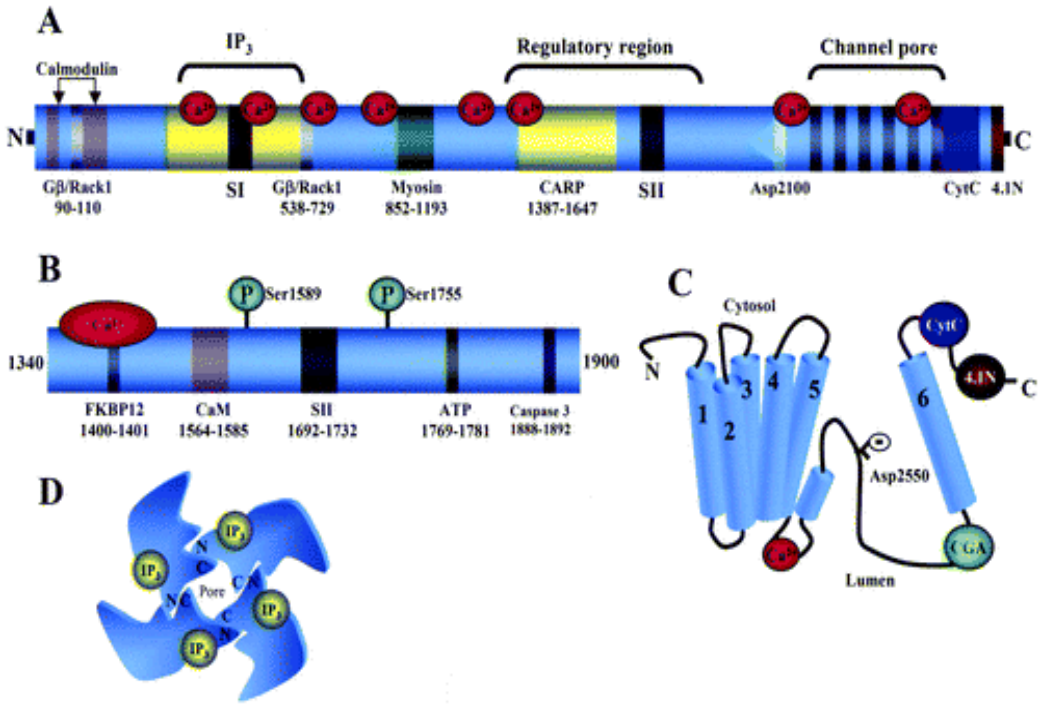


Figure 1.8: Structural organization of the IP3 receptor

The IP3 receptor is a ~310 kD transmembrane receptor that is located primarily in the endoplasmic reticulum and mediates calcium release from intracellular calcium stores. In A and B, the linear view of the receptor shows the specific domains and, more importantly, the known binding sites which are important for IP3 receptor regulation. In panel C the 3-Dimentional view shows the structural organization and functional make-up of the IP3 receptor, as it would be inserted into the membrane. Domains 1-5 make up the pore, and domain 6 constitutes the regulatory C-terminus. Panel D provide a view of the IP3 receptor looking down the pore showing the point at which IP_3 binds in relation to the pore opening (105).

Specific Aims

Aim1: To determine the role of Notch1 in regulating Nur77-induced apoptosis

As described above, over-expression of Nic in D011.10 cells was shown to cause a decrease in activation-induced apoptosis. Studies from our lab suggested that Nic interacts directly with Nur77 and that this interaction may contribute to the decrease in apoptosis seen in this model. Yet another group has shown that Nic and Nur77 interact directly and that this interaction leads to a change in sub-cellular localization and a subsequent decrease in apoptosis. The goal of this first aim is to determine the mechanism by which Nic inhibits apoptosis, specifically its involvement with Nur77. Of significant relevance to this aim is the possible application of this mechanism in the development of T-cell Acute Lymphoblastic Leukemia (T-ALL), as this model might provide additional insight into the mechanisms of how Nic acts as an oncogenic factor.

Aim2: To determine if changes in IP3 receptor isoforms accompany thymocyte negative and/or positive selection.

Calcium fluxes are known to be vital in the induction of negative selection. Work conducted by others also noted differences in calcium fluxes between thymocytes undergoing positive versus negative selection. The goal of the second aim is to determine if changes in IP3 receptor isoforms occur during the induction of negative and positive selection. Also of note is the possible shared interaction of IP3R and Nur77 with members of the Bcl-2 family, specifically Bcl-2. Elegant

experiments have provided data showing that the intact interaction between IP3R and Bcl-2 is anti-apoptotic (88,89). Using a competing peptide that interferes with this interaction leads to increased calcium and subsequent increases in apoptosis.

CHAPTER II

ACTIVATED NOTCH1 REGULATION OF PRO-APOPTOTIC PROTEIN NUR77: IMPLICATIONS FOR LEUKEMIA

Introduction

It is widely accepted that activating mutations of genes encoding the Notch family of transmembrane receptors, specifically Notch1, are associated with oncogenic transformation. Previous data from our lab has shown that an active form of Notch1 (Nic) provides a protective effect against apoptosis in D011.10 T cells; and that this effect may be attributed to Nic binding the pro-apoptotic protein Nur77. Nur77 is an immediate early gene that is upregulated during both negative selection of thymocytes and activation induced apoptosis in D011.10 T cells. Nur77 upregulation is tightly regulated and requires MEF2D, NFAT, and the co-activator, p300, to effectively respond to apoptotic stimuli. In this report we show that Nic has the ability to interfere with the induction of transcription of Nur77, and that this interference is directly related to the inability of p300 to bind the Nur77 promoter in the presence of Nic. We also show that blocking Notch activation through gamma secretase inhibitor or siRNA directed against Notch1 in T cell acute lymphoblastic leukemia (T-ALL) cell lines restores Nur77 upregulation in response to stimuli. These observations support a model in which activating mutations of Notch1 during thymocyte development inhibit the upregulation of a crucial pro-apoptotic molecule.

Materials and Methods

Cell Culture, and Transfections

Jurkat, HPB-ALL, and DND41 cells (kindly provided by Dr. Jon Aster) were cultured in RPMI 1640 media with 20% Fetal Bovine Serum (FBS) (Lonza, Switzerland), 100 U/mL of Penicillin and Streptomycin (Lonza, Switzerland) at 37˚C in a 5% CO_2-humidified incubator. 293T and D011.10 cells were cultured in RDG complete media with 10% FBS (Lonza, Switzerland), 100 U/mL Penicillin and Streptomycin (Lonza, Switzerland) at 37˚C in 7% CO_2-humidified incubator. 293T cells were transfected with Fugene 6 reagent (Roche, Germany) at a ratio of 1 ug of DNA to 3 uL of reagent per the supplier's instructions. Stimulation of DO11.10 cells was performed using 10 nM PMA and 500 nM Ionomycin in DMSO as previously described (39).

Immunoblotting

Gel preparations and protein transfers were done as previously described (39). For immunoblotting, primary antibodies were diluted 1:1000 or as indicated by the manufacturer, using Horseradish Peroxidase (HRP) linked secondary antibodies at a dilution of 1:5000. Detection was performed using Enhanced Chemiluminescence (ECL). The following antibodies were used: β-Actin (Sigma, St.Louis, Missouri), Notch1 (Santa Cruz, Santa Cruz, CA), cleaved Notch1 (BD Biosciences, San Diego, CA), anti-HA (Abcam, Cambridge, MA), anti-myc

(Abcam, Cambridge, MA), anti-p300 (Abcam, Cambridge, MA), and anti-GFP (Abcam, Cambridge, MA). Densitometry of Western blots was done using Image J software and is shown as the average of 3 independent experiments related to β-Actin as an internal control. Statistics were done using Microsoft Excel by performing standard Student's *t*-tests.

Luciferase Assays

293T were transfected with 1 ug of a Nur77 promoter-driven (a kind gift from Dr. Eric Verdin) firefly luciferase plasmid and 25 ng of pRL Renilla luciferase, pCMV-p300, pEGFP-NICD, pEGFP-NICD-NES, and pEGFP-NICD-ΔANK as described. D011.10 and HBP-ALL cells were electroprorated using the Amaxa Nucleofector system (Lonza, Switzerland) using kits T and V, programs O-001 and T-018 respectively. Reporter gene analysis was performed using the Promega Dual Luciferase Reporter Assay System (Promega, Madison WI). The luciferase activity associated with each sample was normalized to Renilla luciferase and calculated into % fold activity as described by the manufacturer (Promega, Madison WI). Graphs were done using Microsoft Excel and are representative of at least 3 independent experiments.

Retroviral and lentiviral infection

Retroviral particles were produced by transfecting 293T cells as described (REF) using the pEco packaging vector along with pBabe-puro-NICD, MIG-R1-NICDΔANK (a kind gift from Dr. Warren Pear), or pBabe-puro-NICD-NES. 24

and 48 hrs after transfection retroviral supernatants were treated with Fugene 6 reagent at a ratio of 6ul of Fugene to 1ml of supernatant and co-cultured with DO11.10 cells. Cells infected with the various pBabe constructs were selected and maintained in media containing 3ug/mL of puromycin. Cells infected with the MIGR1 constructs were diluted in 96-well plates to isolate single cell GFP positive clones; the positive single cell clones were then pooled to make a polyclonal population.

Transfecting 293T cells with pCMV-Delta 8.9 and VSV-G along with pLKO-empty or pLKO-hNotch1 produced lentiviral particles. Fugene was used at a ratio of 7:1 and Delta 8.9 was used at a ratio of 5:1 with VSV-G. 48 and 60 hours after transfection Lentiviral supernatants were supplemented with 8ug/mL of polybrene were titrated at multiple ratios by co-culture with T-ALL cell lines.

Constructs

All constructs used for experiments were purified using the Qiagen endo-free Maxi-prep kit. pBabe-NICD-myc, pBabe-NICD-R2202 (MT), pBabe-NICD-NES, MIG-R1-NICDΔANK, pEGFP-NICD, pEGFP-NICD-NES, pEGFP-NICDΔANK, pLKO-hNotch1, pcDNA-NICD-myc, pEco, pCMV-delta 8.9, and VSV-G have been described (38 - 40). pCMV-p300-HA was purchased from Addgene.

Antibodies

Mouse ChIP grade anti-p300, Goat anti-GFP, and Goat Anti-myc antibodies were purchased from Abcam (Cambridge, MA). Rabbit anti-HA, Rabbit anti-Notch1, and Rabbit anti-GFP were purchased from Santa Cruz (Santa Cruz, CA). Mouse anti-β Actin and Mouse anti-cleaved Notch1 were purchased from BD Biosciences (San Diego CA). The mouse anti-Nur77 antibody is produced in-house as previously described (4).

GSI treatment

Compound E was purchased from Alexis biochemical (Axxora, San Diego CA) prepared in DMSO and is used at a concentration of 100 nM. IL-CHO is kind gift from Abdul Fauq (Mayo Clinic, Fort Lauderdale FL), prepared in DMSO and used at a concentration of 3uM.

Promoter binding assay

The promoter-binding assay was performed as described previously using the Chromatin Immuno-precipitation kit from Abcam (Cambridge, MA) (17). PCR of DNA products from the promoter binding assay were prepared using previously published primers in a Thermo PCR thermocycler at 95˚ for 1 min, 95˚-58˚-65˚ for 30 cycles at 30 seconds each. The amplicons were then run on a 1% Agarose gel (48,50). Youn et al and Dequiedt et al previously described the

primer sets that were used (48,50). They are labeled as Primer Set 1 and Primer Set 2 respectively.

Primer Set 1 Forward: 5'-AGGGGGAGGAGATCCTGTTC-3'

Reverse: 5'-ATTGACGCAGGGAGCGCGGAT-3'

Primer Set 2 Forward: 5'-AGGACAGACTGGGAAAGGGACAAA-3'

Reverse: 5'-AGGGAGCGCGGATTGTTTGAT-3'

Immunoprecipitation

293T cells were transfected with constructs containing Myc-tagged NICD and HA-tagged p300 were transfected as described above. Transfected cell extracts were immunoprecipitated with goat anti-Myc antibody (Abcam Cambridge, MA) and isotype control antibodies as previously described by Oswald *et al* (33)

Cell Death Assay and Flow Cytometry

Flow Cytometry was performed using an LSRII (Beckon-Dickinson, Mountain View, CA) following the staining protocol provided with the Cell Death Assay Kit (BD Biosciences, San Diego CA).

Results and Discussion

Activated Notch1 expression in DO11.10 cells leads to a decrease in cell death and Nur77 levels

It is well known that Notch protein members regulate the transcription of genes by interacting with a variety of transcriptional regulators, most notably CSL, NF-κB, MAML, and p300 (92,107,108). Early results from our lab suggested that Notch inhibits Nur77-mediated apoptosis at least partly by direct protein-protein interaction (103). Thus, we wanted to further examine the effect of activated Notch1 on Nur77 by closely analyzing Nur77 transcription. We stably-infected the DO11.10 T cell hybridoma with active Notch1 (NICD), NICD lacking the ankyrin domain (ΔAnk), or NICD consisting only of the ankyrin domain and the NLS sequences. This construct is also known as the minimum-transforming domain (MT) as previously described by Capobianco and colleagues (97). The ΔAnk mutation removes the domain responsible for protein complex formation and protein-protein interaction. Previous research done in our lab has provided evidence that Nur77 and NICD interact, and that this interaction may be protective against apoptosis (103). Thus, using both the ΔAnk and the MT constructs allowed us to determine the contribution of the ankyrin domain of NICD to Nur77-induced apoptosis.

DO11.10 cells stably expressing these constructs were generated by retroviral infection followed by selection in the presence of puromycin for a period of 7

days, and continued maintenance in puromycin-containing media. Control DO11.10 cells, infected with the empty vector pBabe, were treated with PMA and Ionomycin (P + I) for 16 hour and showed high levels of cell death when stained with propdium iodide (PI) and subjected to FACS analysis (Fig. 2.1B). As expected from our previous studies (103), over-expression of NICD led to a decrease of cell death in DO11.10/NICD cells induced to undergo apoptosis *via* stimulation with P+I (Fig. 2.1C). The decrease in cell death was dependent on the Ankyrin domain of NICD, since using a mutant version of NICD (ΔAnk) lacking this domain restored cell death in response to stimulation with P+I (Fig. 2.1D). DO11.10 cells over-expressing the MT construct showed levels of cell death comparable to empty vector-expressing cells. (data not shown).

We next determined whether NICD affects Nur77 protein levels in DO11.10 cells. We performed western blot analysis on empty vector, NICD and NICDΔAnk infected DO11.10 cells. Nur77 expression in NICD over-expressing cells was noticeably decreased compared to empty vector control lysates (Fig. 2.2A). In contrast, NICDΔAnk containing cells showed no decrease in Nur77 protein levels compared to control DO11.10/empty vector cells (Fig. 2.2A). To determine if NICD affects Nur77 transcription, NICD over-expressing DO11.10 cells were electroporated with a Nur77 promoter luciferase construct (20). NICD over-expressing DO11.10 cells showed decreased Nur77 promoter activation compared to controls (Fig. 2.2B). These observations suggest that NICD regulates the expression of the pro-apoptotic protein, Nur77.

Activated Notch1 over-expression represses MEF2C and MEF2D enhancement of Nur77 promoter and MEF2 responsive promoter

Studies in myocytes and other cells have shown that activated Notch1 has the ability to inhibit members of the MEF2 family of transcription factors. These same groups have shown that this inhibition can be either through direct interaction with MEF2 or through another common co-activator Mastermind (MAML). To determine if the inhibition of the Nur77 promoter could in fact be through Nic regulation of MEF2 transcription factor luciferase assays were performed using the pNur77-luc and MEF2 Response element reporter (MRE) constructs with MEF2C or MEF2D in the presence of exogenous Nic. In both cases a substantial decrease in promoter activity was observed (Fig 2.3+2.4). To further determine if this interaction could be involved similar experiments were done using a luciferase construct that was under to control of MEF2 response elements and as expected we observed decreases in activity in the presence of Nic (Fig.2.5). As shown earlier Nic decreases the activity of the Nur77 promoter in DO11.10 cells stimulated to undergo apoptosis. To show that this decrease could be the result of MEF2 regulation we performed a similar electroporation and luciferase assay with the MRE-luc construct and observed a similar decrease in activity (Fig.2.6). As mentioned MEF2C has been published to interact directly and indirectly with Nic, we performed immunoprecipitations as were previously published but were unable to repeat the results seen by other groups. It is also important to note that though MEF2C was shown to up regulate the Nur77

promoter it is unclear whether this isoform of MEF2 exists in our proposed DO11.10 model. Specific antibodies that recognize the unique isoforms do exist, but showed significant background, which made it nearly impossible to make a clear conclusion. It has been well established that MEF2D is vital in the upregulation of Nur77, though no documented direct interaction exists between Nic and MEF2D. Upon further examination of the literature it became clear that another common co-activator could be involved in the repression that were we observing in the presence of Nic, another such common co-activator was p300.

Activated Notch1 repression of MEF2D enhancement of both the Nur77 and MRE promoters is p300 dependent

To access the importance of p300 in our MEF2 enhancement of the Nur77 promoter, we co-expressed p300 in the same 293T system as described in detail earlier. Interestingly we observed differences in the dependence of p300 between MEF2C and MEF2D, MEF2C showed increased activity in the presence of p300 but it was completely abolished by Nic (Fig.2.8). MEF2D however showed restored activity in the presence of exogenous p300 in spite of the presence of Nic, similarly this restoration was seen to be dose dependent (Fig.2.7+2.9). These results provide interesting differences between isoforms and would suggest that regardless of our abilities to provide details, that MEF2C is inhibited by a different p300 independent mechanism. As mentioned earlier MEF2D is thought to be the predominant MEF2 involved in the upregulation of Nur77 (96,100).

Also work from other labs had established that Nic transcriptional regulation through p300 is a valid mechanism for inhibiting potential target genes (94,106). The co-activator p300 hence becomes the possible common factor that could be involved in Nic inhibition of Nur77 promoter activation.

Activated Notch1 over-expression represses p300-enhancement of Nur77 promoter activity

As previously determined by other groups, Nur77 transcription is regulated by several transcriptional modulators including the co-activator p300 (48). Additionally, NICD can sequester p300 through direct interaction, preventing p300-mediated induction of target genes (108,109). To determine if NICD regulates p300-enhanced Nur77 expression, we performed luciferase assays in 293T cells cotransfected with full-length Nur77 promoter-driven luciferase, together with constructs containing p300, and WT and mutant NICD constructs. In earlier experiments (shown in Fig. 2.1C) NICD was shown to reduce Nur77 expression whereas ΔANK displayed no inhibitory effect on Nur77 protein expression. As a control, we used a NICD expression construct tagged with Nuclear Export Sequences (NES). NICD-NES is known to localize solely to the cytosol and has been used previously in our lab to determine the cytosolic contributions of NICD (52). This construct allowed us to determine whether nuclear localization is required for NICD mediated repression of Nur77 expression.

As expected, NICD over expression reduced p300-dependent enhancement of Nur77 promoter activity (Fig. 2.11A) in a dose-dependent manner (Fig. 2.11B). 293T cells were also co-transfected with the mutated Notch construct, NICDΔAnk, which lacks the Ankyrin domain, and NICD-NES, which is constitutively excluded from the nucleus. NICD-induced repression of p300-induced Nur77 promoter activity was abrogated in the absence of the ankyrin domain (Fig. 3C), and the repression also required NICD nuclear localization since NICD-NES expression was unable to repress Nur77 promoter activity (Fig. 2.11D). DO11.10 cells infected with NICD, as well as control DO11.10 cells were electroporated with the Nur77 promoter luciferase construct to assess Nur77 activity. These cells were subsequently stimulated with P+I and, similar to the experiments in 293T cells, showed a decrease in Nur77 promoter activity in the presence of NICD (Fig. 2.2B). These results provided direct evidence that NICD acts as a transcriptional repressor of the Nur77 promoter, and this likely involves the co-activator p300.

Activated Notch1 over-expression leads to decreased p300 binding of the Nur77 promoter

Oswald and coworkers showed that NICD binds directly to p300 mediated in part by the E/P region of NICD, a region located near the ankyrin domain (106). Therefore we performed immunoprecipitation of activated Notch1 and p300 in 293T cells, which showed a direct binding of NICD to p300 (Fig. 2.12A). NICD and p300 over expression in 293T cell lead to both co-localization and a

more diffuse patterning of NICD in the nucleus (Fig. 2.13A) Results from other groups concluded that NICD could interfere with p300 binding to specific promoters (32). We performed promoter-binding assays to determine the effects of NICD on the ability of p300 to access the Nur77 promoter and regulate its activity. Youn *et al*. showed that p300 and NFAT bind MEF2D on the Nur77 promoter (48). They also provided evidence that p300 binding to this nuclear complex is necessary for maximal promoter activation (48). We transfected 293T cells with a construct containing the Nur77 promoter in the presence of p300 alone or with constructs containing NICD and NICD-NES. The cells were harvested 16 hours later for chromatin immunoprecipiation, followed by PCR analysis using primers that are known to encompass the reported p300-binding region (48,50). As expected, p300 bound the Nur77 promoter (Fig. 2.14A). In contrast, expression of NICD resulted in a substantial reduction of p300 binding to the Nur77 promoter (Fig. 2.14A). No reduction in p300 binding was observed in cells transfected with NICD-NES, suggesting once more that NICD nuclear localization is required for its interaction with p300 (Fig. 2.14B) and that this interaction inhibits p300 binding to the Nur77 promoter.

To determine whether the same mechanism was observed in a model of Nur77-induced apoptosis, the same promoter-binding assay was performed in DO11.10 cells electroporated with a construct containing the Nur77 promoter and then subjected to ChIP analysis. The data from these experiments confirmed the results seen in 293T cells (Fig. 2.14C). Taken together, these results suggest that NICD represses p300-dependent Nur77 transcription by decreasing the capacity

of p300 to access the Nur77 promoter. Additionally these results provide evidence that this mechanism is conserved in an *in vitro* model of Nur77-dependent activation-induced apoptosis.

Activated Notch1 represses Nur77 in T cell acute lymphoblastic leukemia cells

Activating mutations in Notch1 correlate with development of T cell acute lymphoblastic leukemia (T-ALL) (95,96,100). T-ALL is a neoplasm characterized by circulating immature double positive lymphoblasts/thymocytes. Notch1 is essential for T cell development but is conspicuously absent during negative selection, a period that characteristically involves the upregulation of the pro-apoptotic protein Nur77. The T-ALL cell lines are known to over-express NICD. To determine if T-ALL cells have defect in Nur77 upregulation, cells were stimulated with P+I alone or in the presence of the gamma secretatase inhibitor (GSI) IL-CHO or Compound E. After 2 hours, expression of Nur77 was determined by immunobotting. Stimulating T-ALL cells with P + I alone did not lead to an increase in Nur77 protein levels (Fig. 2.15A+B). However, repressing Notch activation with GSI resulted in increased levels of Nur77 (Fig. 2.15A+B). We next sought to determine whether T-ALL cells had a similar decrease in Nur77 promoter activation as other NICD over-expressing cell types we tested. To do this, we transfected T-ALL cell lines by electroporation with a full-length Nur77 promoter luciferase construct. The transfected cells were then treated with GSI and assayed for activity after 16 hours. GSI treatment led to an increase in

luciferase activity after treatment with P + I (Fig. 2.15C+D). As an extension of the experiments done in both 293T and DO11.10 cells, we also determined the binding of p300 to the Nur77 promoter in T-ALL cells. Using cells treated as above, we performed promoter-binding assays and determined that treatment of T-ALL cells with GSI leads to increased p300 binding to the Nur77 promoter (Fig. 2.15E). To further determine whether this effect was NICD-dependent, T-ALL cells were lentivirally infected with shRNA constructs that targeted Notch1, resulting in reduced levels of the protein (Fig. 2.15F). Knockdown of Notch1 in T-ALL cell lines led to rapid cell death (unpublished results), and the increased expression of Nur77 (Fig. 2.15F). These results show that the constitutive activation of Notch in T-ALL cells prevents apoptosis, at least partially, through the negative regulation of Nur77. Furthermore, this negative regulation is, in part, due to the transcriptional down regulation of Nur77 *via* NICD-directed inhibition of p300 promoter binding.

Discussion

During T cell development, expression of Nur77 in thymocytes and immature T-cells is associated with negative selection. Past research on the mechanism of action of Nur77- induced apoptosis has demonstrated that cytosolic localization of Nur77 is vital for its pro-apoptotic program (10,45). More recently, work from our lab provided convincing data detailing the phosphorylation events involved in the pro-apoptotic function of Nur77 (45). Importantly, these data clearly show that Nur77 cytosolic localization is sufficient to induce apoptosis in DO11.10 cells, an *in vitro* model of negative selection (45). Several studies have suggested a mechanism of inhibition of Nur77 induced apoptosis through direct interaction with the activated form of Notch1 (41,103). In agreement with these data, we show here that over-expression of NICD in DO11.10 T cells decreases cell death, and that this decrease involves a subsequent decrease in a known pro-apoptotic molecule, Nur77. Previous cell death studies in Hela cells using NICD and EBNA2, a NICD like molecule, showed that the observed anti-apoptotic effect was *via* direct protein-protein interaction of Nur77 and NICD or EBNA2 and this resulted in Nur77 nuclear localization (41). Indeed, we observed that NICD and Nur77 directly interact *via* immunoprecipitation and that this interaction is dependent on the ankyrin domain of NICD and the DNA binding domain (DBD) of Nur77 (data not shown).

However, in this report we present evidence supporting another novel mechanism whereby NICD suppresses Nur77 by limiting p300 access to the Nur77 promoter. This, in turn, leads to a decrease in Nur77 activity. It is possible

that Notch blocks Nur77-induced apoptosis both by sequestration of p300, thus blocking transcription of Nur77 and by retention of Nur77 in the nucleus. Masuda *et al*. has shown previously that NICD interferes with TGF-β signaling through a sequestration of p300 from Smad3 (108). This same study also demonstrated that addition of excess p300 had the ability to restore Smad signaling in the presence of NICD. Similarly, they also showed that down regulation of Notch1 *via* shRNA restored sensitivity to TFG-β in their model (108). Although others have shown that NICD interferes with signaling pathways by p300 sequestration, the data in this report are the first to implicate NICD interference with Nur77 expression and activity through p300 sequestration. Our results are quite similar to those of Masuda *et al.* in that NICD over-expression sequesters p300 from the Nur77 promoter, perhaps by competing with its known association with the NFAT/MEF2D complex (48,50).

Activating mutations of Notch1 are known to be present in over 50% of cases of T-ALL (103). In these instances, T-ALL cells display varying sensitivity to GSI. Activating mutations in Notch1 are associated with transformation and work from other labs has demonstrated the importance of nuclear localization of NICD for its transforming abilities (95-97,100). Additionally, these activating mutations of Notch1 during T-ALL development have been shown to influence multiple cellular processes including upregulation of c-myc and NF-κB among others (95,107). In this report, we also have found that NICD sequestration of p300 has relevance in T-ALL cell lines. We provide evidence that after treatment with GSI or Notch1 shRNA, Nur77 levels increase in response to stimulation. Our

data suggests the possibility that Notch1 mutations resulting in constitutively active NICD could inhibit Nur77 expression and subsequent induction of apoptosis facilitating the development of T-ALL. Interestingly, NICD-mediated upregulation of Deltex1 has also been shown to interfere with p300-mediated gene regulation specifically in glucocorticoid-induced apoptosis (109). While we did not examine Deltex1, it is also possible that a Deltex1-dependent mechanism may be important during T-ALL development. Also of interest is the role of Mastermind during this process, since published reports provide a direct link between Mastermind and p300 (110). Whether Mastermind, in a complex with NICD and CSL, also selectively recruits p300 from the Nur77 promoter remains to be elucidated.

It is well established that Notch1 levels are decreased during thymocyte negative selection. It is conceivable that activating mutations of Notch1 during negative selection could lead to increased expression of NICD, suppressing expression of Nur77, a known pro-apoptotic regulator of negative selection, helping to facilitate development of T-ALL. The data provided in this report support such a model.

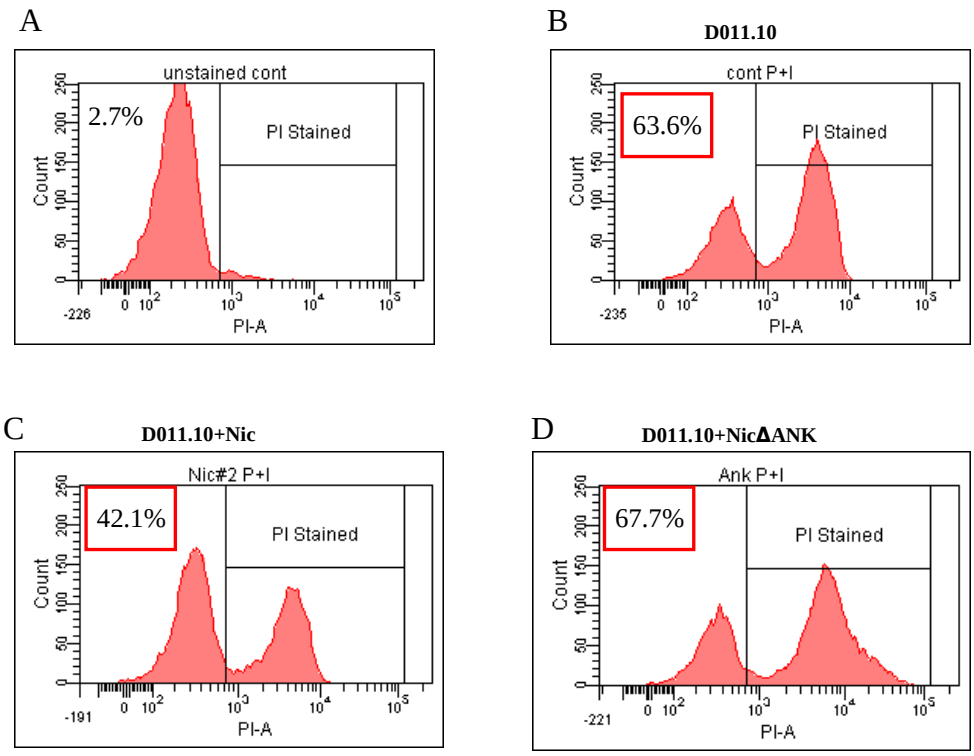


Figure 2.1: Intracellular Notch1 (Nic) over expression decreases activation - induced cell death in DO11.10 cells

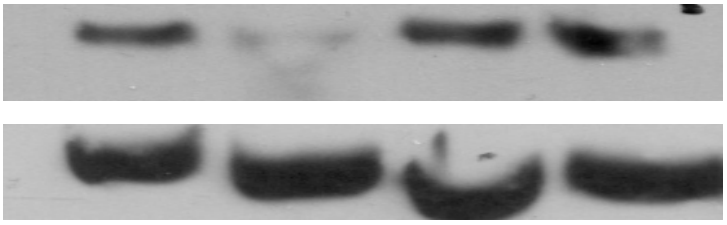
1A) pBabe-empty vector infected DO11.10 cells stimulated with 10nM PMA and 500 nM Ionomycin for 16 hours and used as an unstained control were analysis by Flow Cytometry on an LSRII.

1B) pBabe-empty vector infected DO11.10 cells stimulated with 10nM PMA and 500 nM Ionomycin for 16 hours and stained with Propdium Iodide were analysis by Flow Cytometry on an LSRII.

1C) pBabe-Nic infected DO11.10 cells over expressing exogenous Nic stimulated and treated as in 1A.

1D) MigR1-NicΔANK infected DO11.10 cells over expressing exogenous NicΔANK stimulated as in 1A+1B.

A Empty Vector Nic MT NicΔANK



B

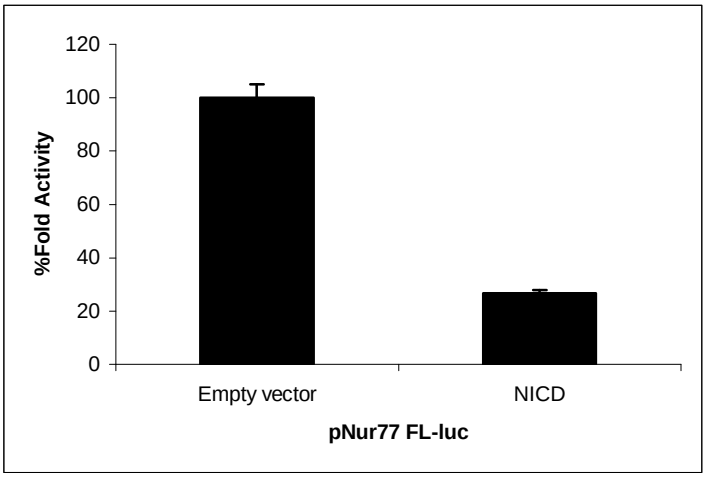


Figure 2.2: Nic over expression in DO11.10 cells results in decreased Nur77 expression in response to stimulation

2A) Western blot of DO11.10 cells infected with pBabe-empty, Nic, MT, or NicΔANK showing Nur77 expression after stimulation with 10nM PMA and 500 nM Ionomycin. Protein lysates were run on an 8% SDS-PAGE gel Actin used as an internal control.

2B) Luciferase activity of the Nur77 promoter in DO11.10 or DO11.10/Nic over expressing cells stimulated with 10nM PMA and 500 nM Ionomycin 2hr as described in methods.

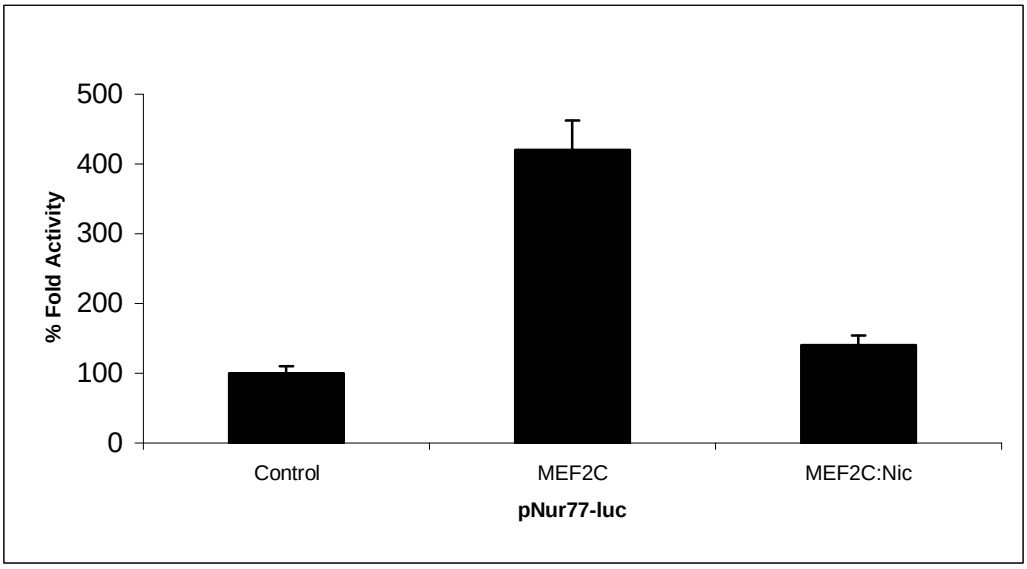


Figure 2.3: Nic over expression in 293T cells inhibits MEF2C enhancement of the Nur77 promoter

2.3A) 293T cells were transfected with MEF2C alone or MEF2C plus equal amounts of Nic. After 24hr cells were processed for luciferase activity as described in the methods.

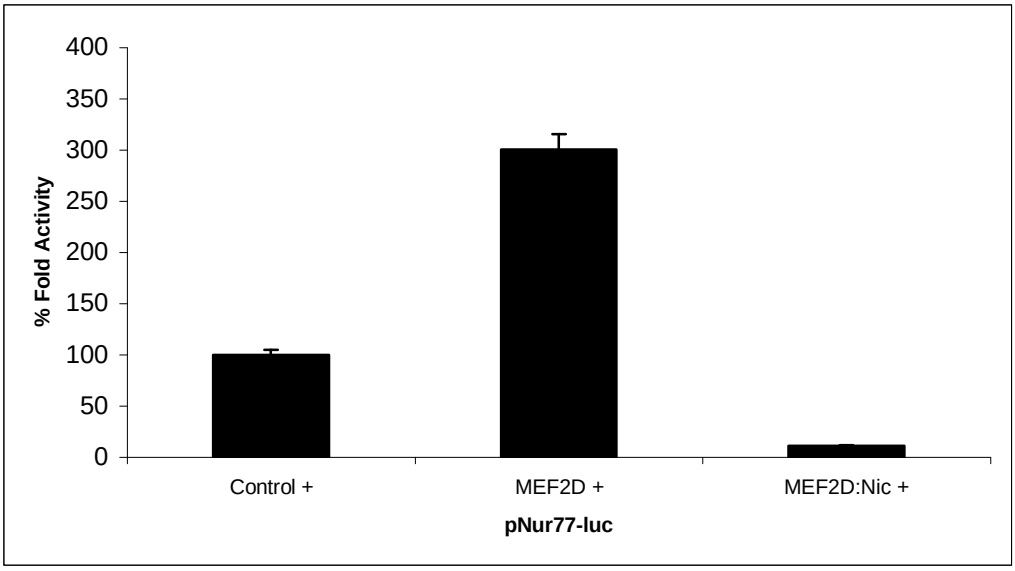


Figure 2.4: Nic over expression in 293T cells inhibits MEF2D enhancement of the Nur77 promoter

2.4A) 293T cells were transfected with MEF2D alone or MEF2C plus equal amounts of Nic. After 24hr cells were stimulated with PMA/Ionomycin and processed for luciferase activity as described in the methods.

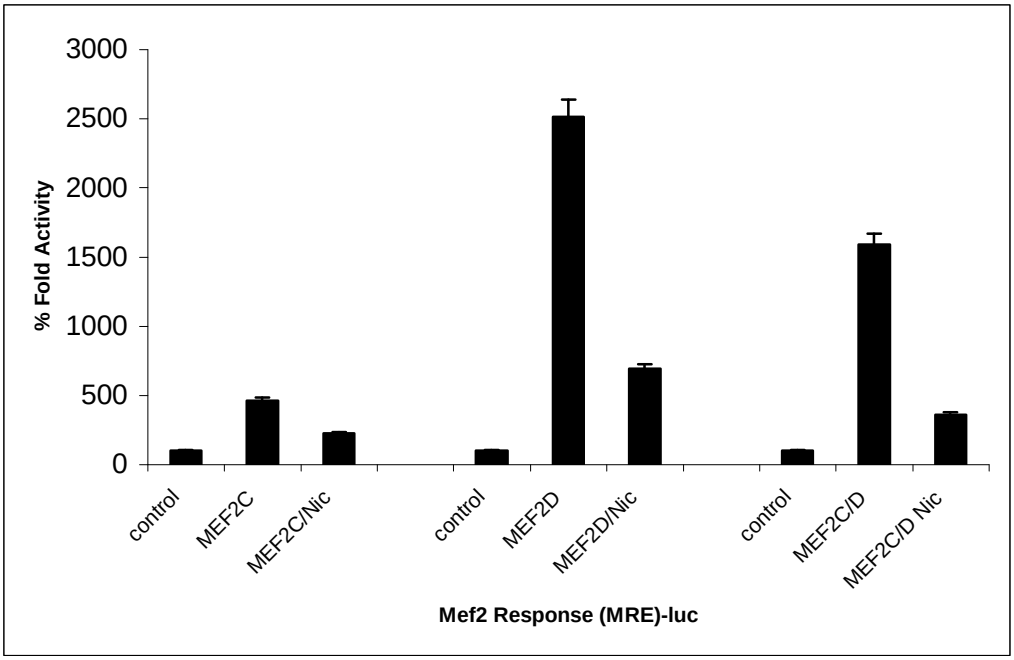


Figure 2.5: Nic over expression in 293T cells inhibits MEF2C and MEF2D enhancement of MEF2 Responsive Element (MRE) activity

2.5A) 293T cells were transfected with MEF2C, MEF2D, MEF2C/MEF2D plus equal amounts of Nic. After 24hr cells were processed for luciferase activity as described in the methods.

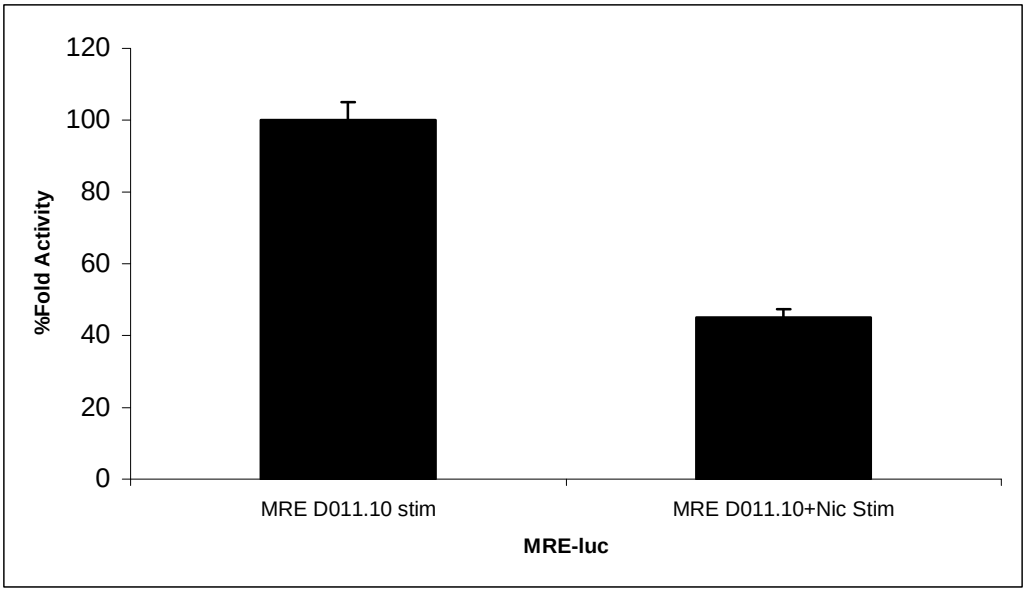


Figure 2.6: Nic over expression in DO11.10 cells inhibits MEF2 response element upregulation during PMA/Ionomycin stimulation

2.6A) DO11.10 or DO11.10 Nic over expressing cells were electroporated with an MRE-luc construct as described in detail in the methods section. After 24hr cells were stimulated with PMA/Ionomycin for 2hr and subsequently processed for luciferase activity as described in the methods.

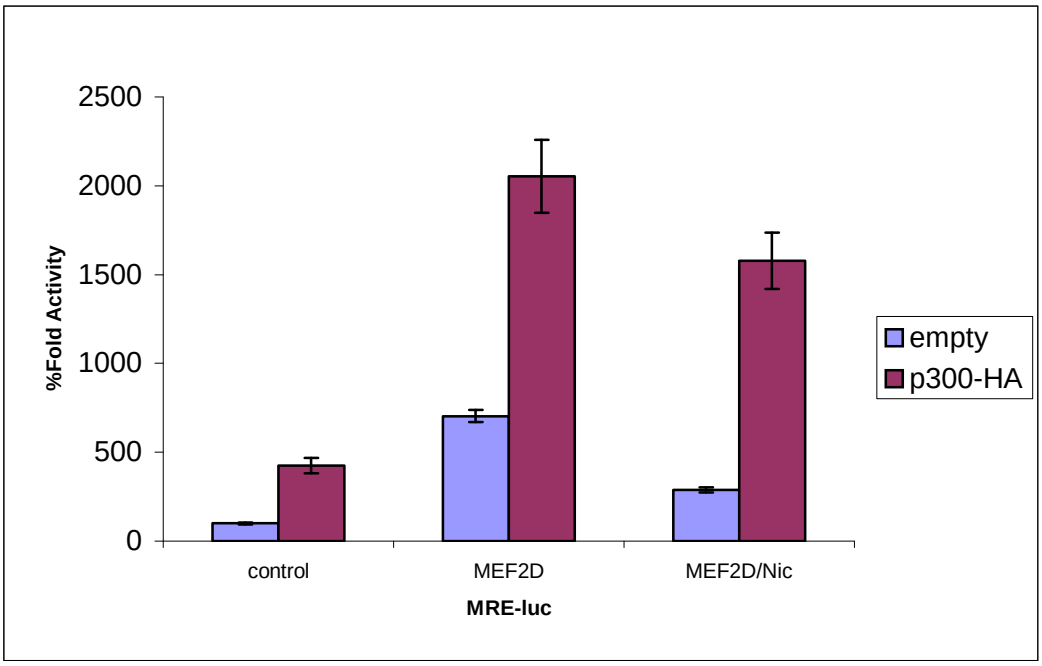


Figure 2.7: Nic inhibition of MEF2D is p300 dependent

2.7A) 293T cells were transfected with MEF2D or MEF2D plus Nic in the presence or absence of exogenous co-activator p300. After 24hr cells were processed for luciferase activity as described in the methods.

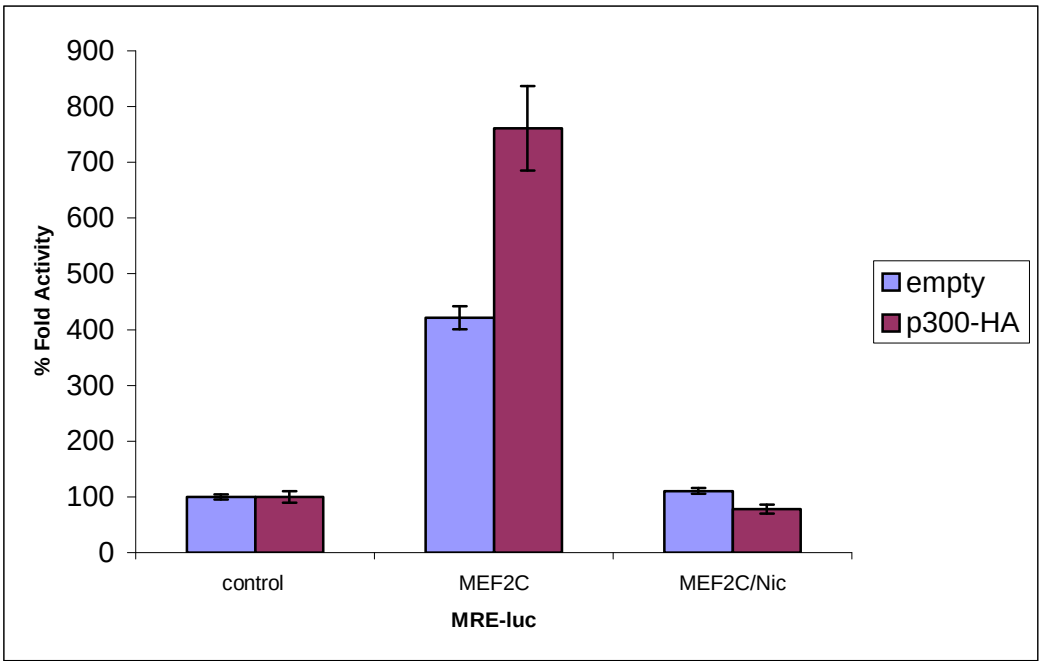
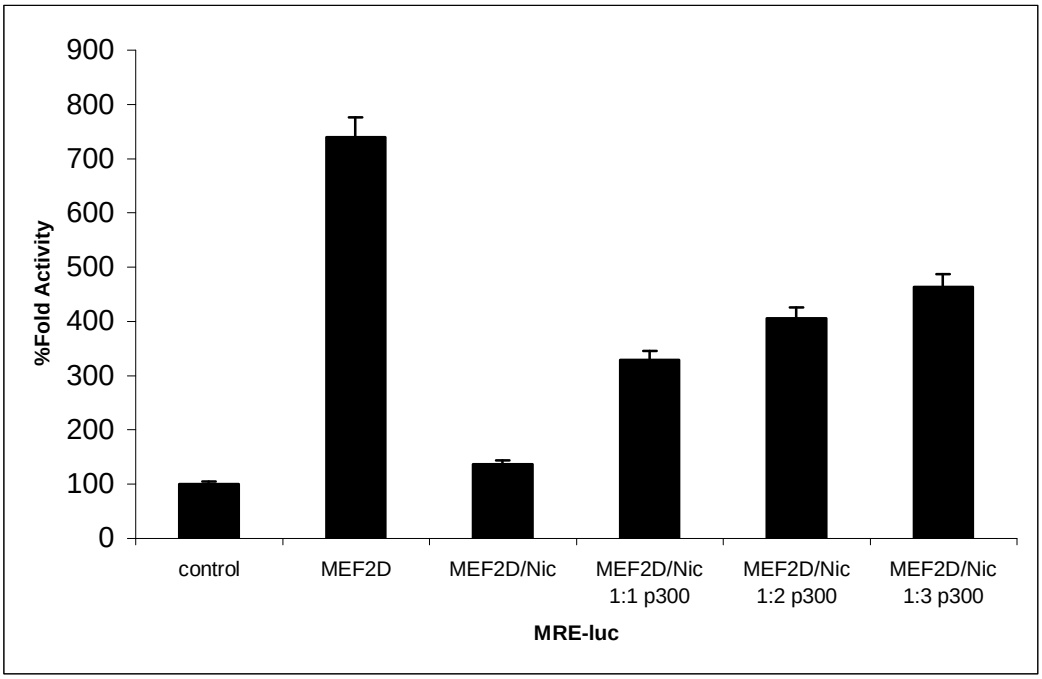


Figure 2.8: Nic inhibition of MEF2C is p300 independent

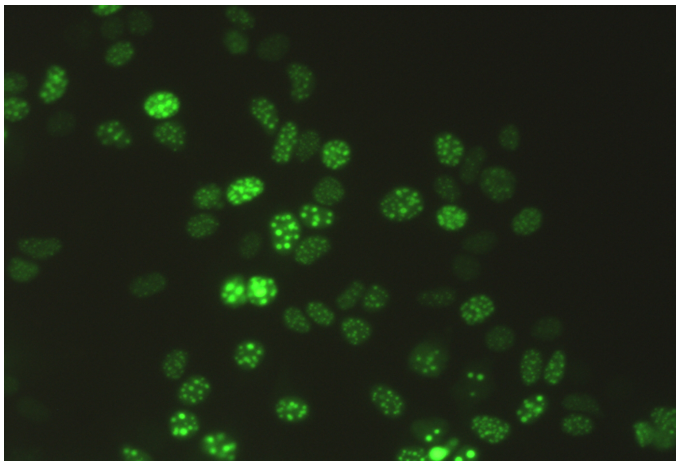
2.8A) 293T cells were transfected with MEF2D or MEF2D plus Nic in the presence or absence of exogenous co-activator p300. After 24hr cells were processed for luciferase activity as described in the methods.



<u>Figure 2.9:</u> Nic inhibition of MEF2D can be rescued by increasing amounts of p300

2.9A) 293T cells were transfected with MEF2D or MEF2D plus Nic in the presence or absence of exogenous co-activator p300 in increasing p300/Nic ratios. After 24hr cells were processed for luciferase activity as described in the methods

A



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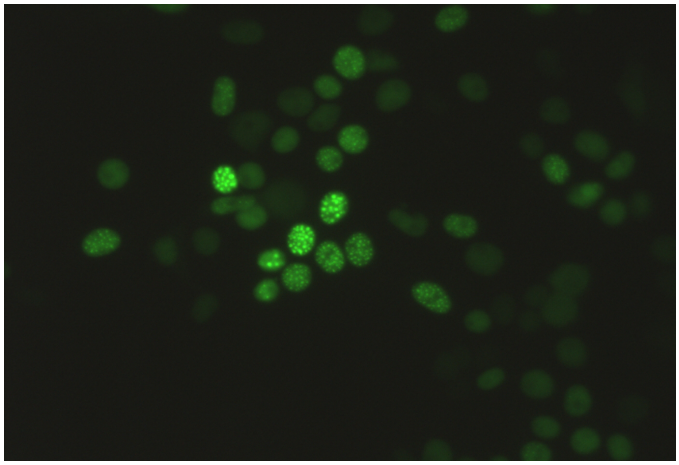

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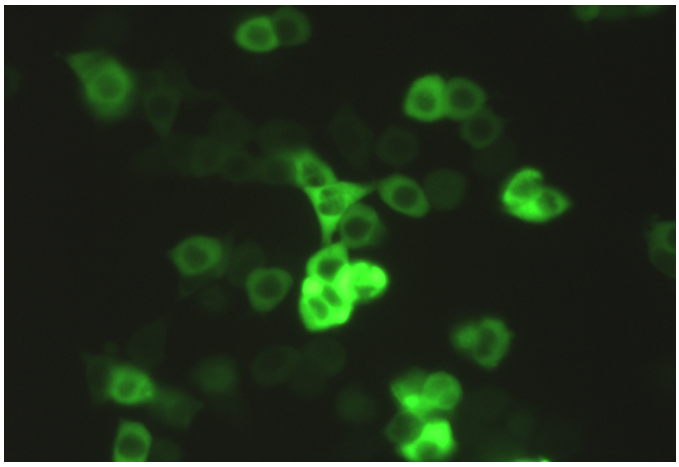


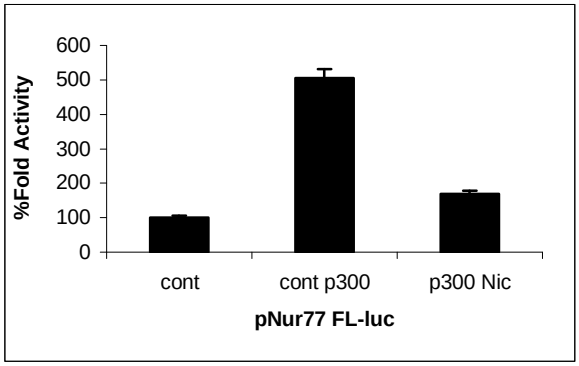
Figure 2.10: Expression and localization of Nic and Nic mutants *in vitro*

2.10A) 293T cells were transfected with pEGFP-Nic showing distinct nuclear localization with characteristic nuclear speckles.

2.10B) 293T cells were transfected with transfected with pEGFP-NicΔANK showing nuclear localization with characteristic nuclear speckles.

2.10C) 293T cells were transfected with pEGFP-Nic-NES showing cytosolic localization with complete loss of nuclear staining.

A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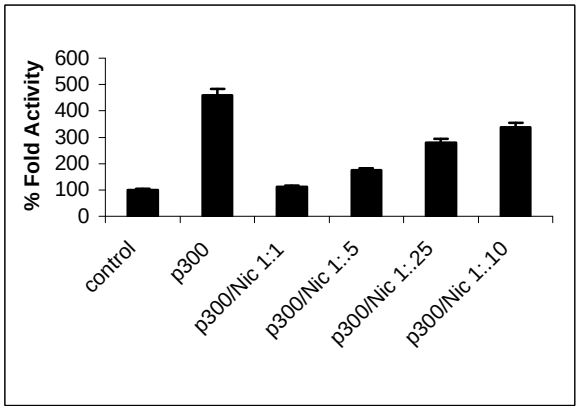
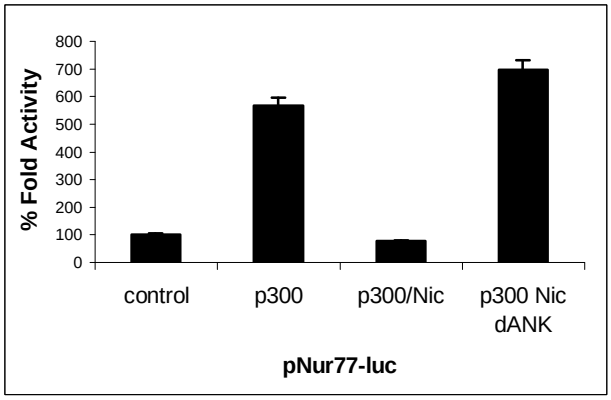
600
500
400
300
200
100
0
cont
cont p300
p300 Nic
pNur77 FL-luc

B


% Fold Activity
600
500
400
300
200
100
0
control
p300
p300/Nic 1:1
p300/Nic 1:.5
p300/Nic 1:.25
p300/Nic 1:.10

C



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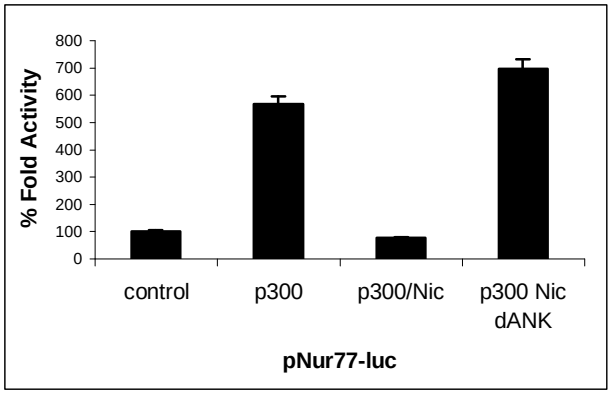


<u>Figure 2.11:</u> Nic over expression reduces p300 enhancement of the Nur77 promoter

2.11A) 293T cells were transfected with pNur77-luc with p300 or p300/Nic as described in methods. Luciferase activity of the Nur77 promoter in the presence of p300 or p300 plus Nic was assayed using a Promega Dual luciferase kit as described by the manufacturer.

2.11B) 293T cells transfected with pNur77-luc with p300 or p300 with various amounts of Nic. Luciferase activity of the Nur77 promoter in response to decreasing amounts of Nic was assayed using a Promega Dual luciferase kit as described by the manufacturer.

2.11C) 293T cells were transfected with pNur77-luc. Luciferase activity of the Nur77 promoter in the presence of p300 alone, p300+Nic, and p300+Nic-NES was assayed using a Promega Dual luciferase kit as described by the manufacturer.

2.11D) 293T cells were transfected with pNur77-luc. Luciferase activity of the Nur77 promoter in the presence of p300 alone, p300+Nic, and p300+NicΔANK was assayed using a Promega Dual luciferase kit as described by the manufacturer.

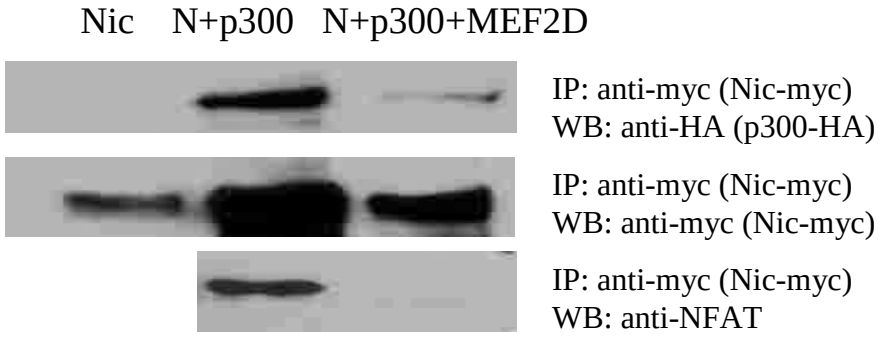


Figure 2.12: Nic specifically binds to p300 *in vitro*

2.12 A) 293T cells were transiently transfected with myc-tagged Nic were also transfected as listed above with HA-p300 or HA-p300 and pCMV-Mef2D. Immunoprecipitation was performed on lysates and run on SDS-PAGE to identify interactions.

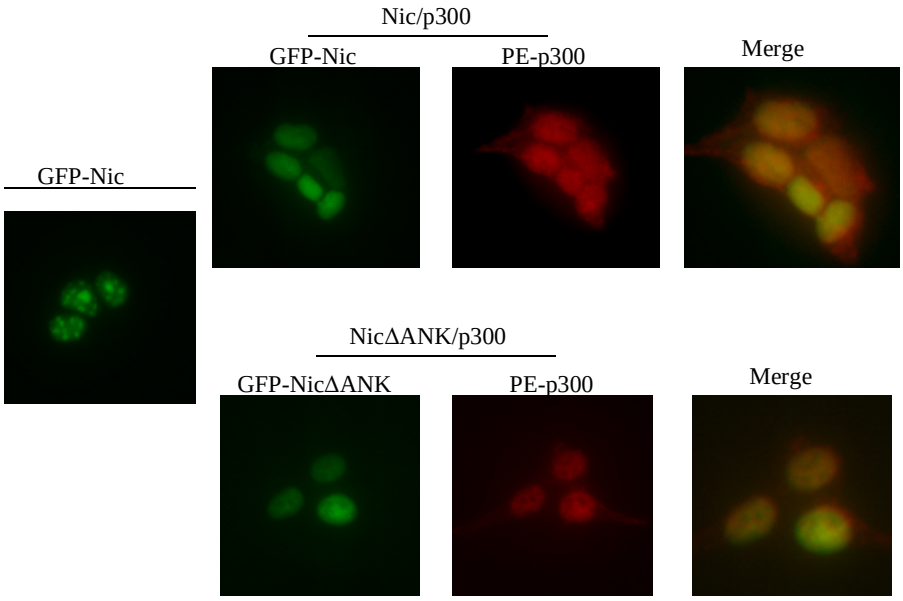


Figure 2.13: p300 over expression changes nuclear distribution of Nic or NicΔANK

2.13A) 293 T cells cultured on sterile cover slips were transfected as in methods with Nic-GFP or NicΔANK with or with HA-p300. After 24 hours cells were fixed using 4% PFA for 5 minutes, treated with 0.2% Triton X PBS for 20 minutes, washed 3 times with PBS, blocked with 1% BSA in 0.2% Triton X PBS and incubated overnight with primary rabbit anti-p300 in blocking buffer. After the O/N incubation, cells were washed three times with PBS and incubated for 1 hour in blocking buffer with PE-labeled anti-rabbit secondary and isotype control. Cover slides containing stained cells were mounted using anti-fade mounting media.

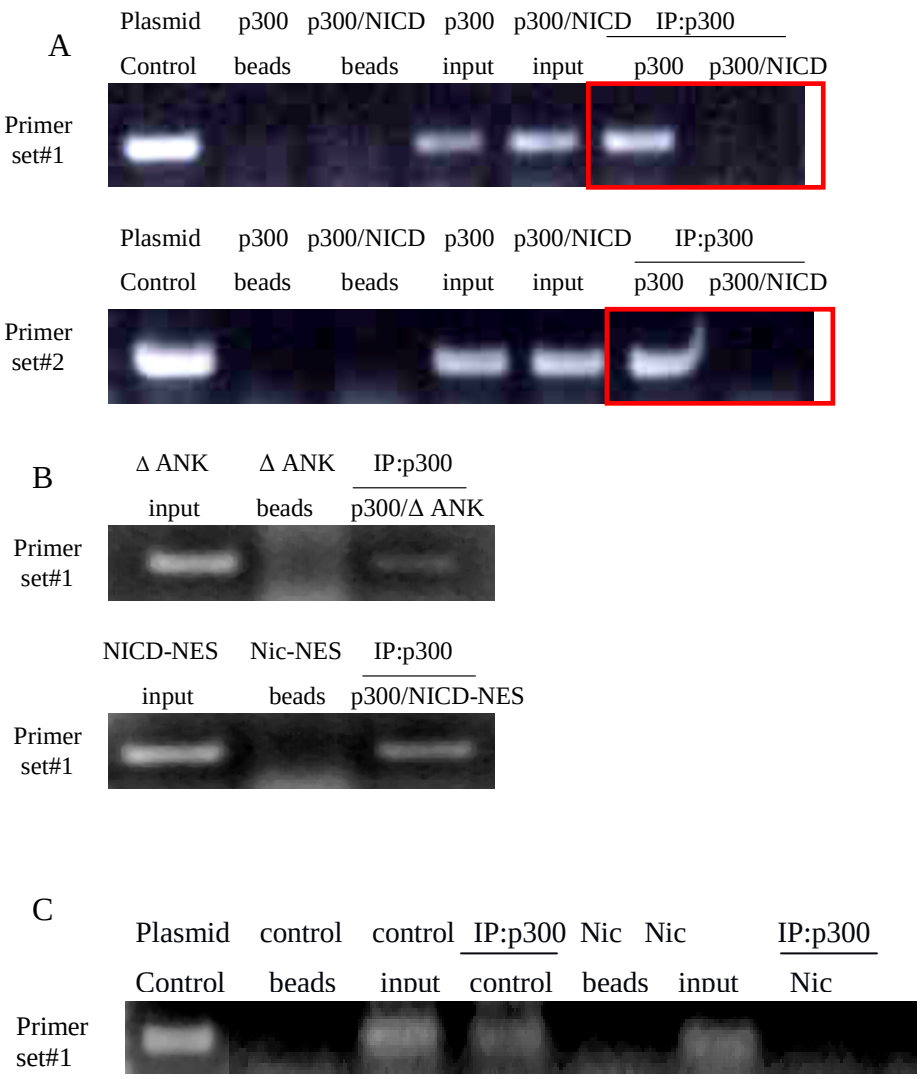


Figure 2.14: Nic over expression reduces p300 binding of the Nur77 promoter

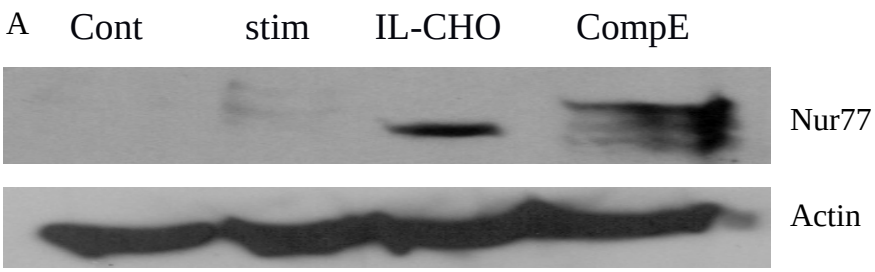
2.14A) Promoter binding assay of the Nur77 promoter construct in 293T cells. 293T cells were transfected with pNur77-luc, p300, or p300/Nic and then subjected to Chromatin Immunoprecipitation as described in methods. PCR was performed using previously published primer sets as described in the methods.

Primers show p300 binding elements of the Nur77 promoter in control sample and a loss of binding in the presence of Nic.

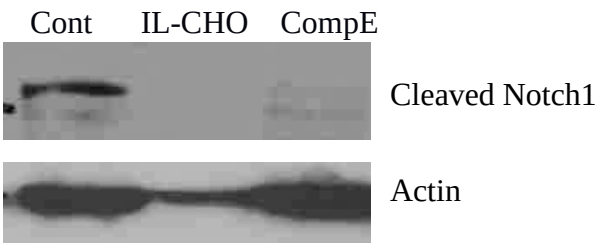
2.14B) Promoter binding assay of the Nur77 promoter construct in 293T cells. 293T cells were transfected with pNur77-luc, p300, or p300/Nic-NES and then subject to Chromatin Immunoprecipitation as described. PCR was performed using previously published primer sets as described in the methods. Primers show p300 binding elements of the Nur77 promoter in control sample and in the sample containing the cytosolic retained Nic-NES.

2.14C) Promoter binding assay of Nur77 promoter construct in DO11.10 cells shows results similar to those in 2.14A.

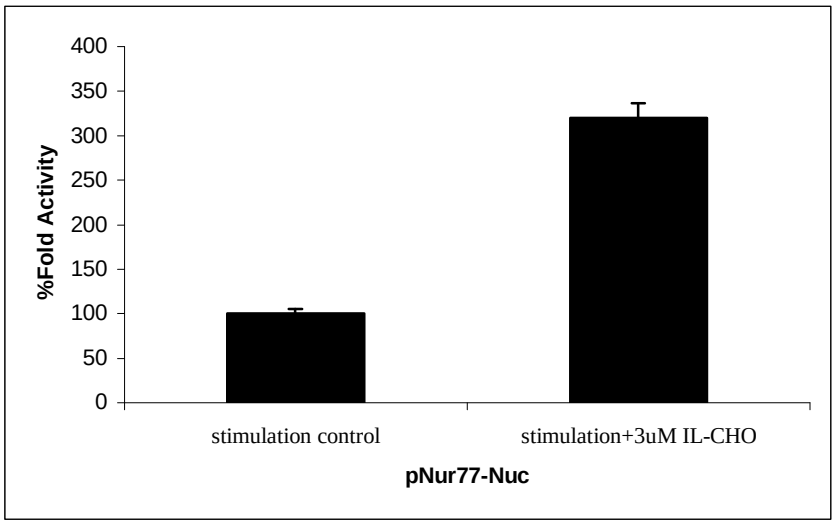
A


Cont
stim
IL-CHO
CompE
Nur77
Actin

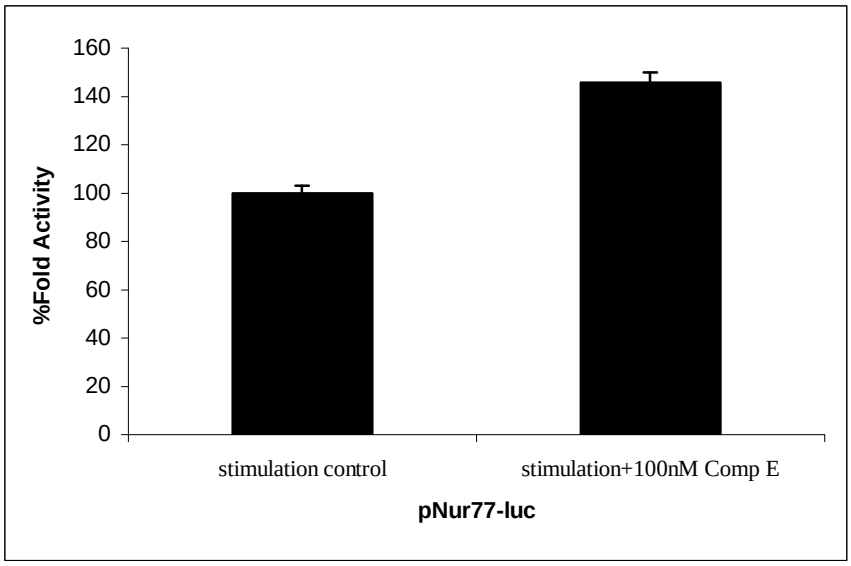
B


Cont
IL-CHO
CompE
Cleaved Notch1
Actin

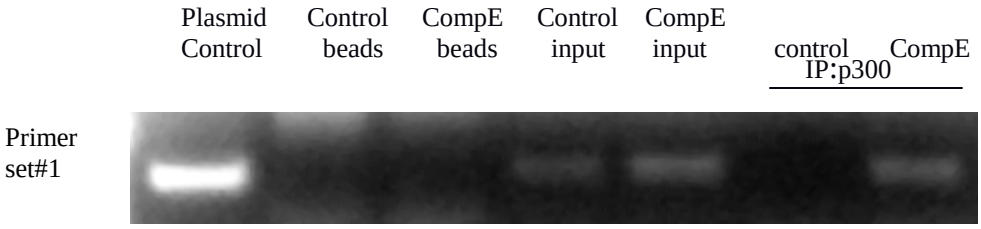
C


400
350
300
250
200
150
100
50
0
%Fold Activity
stimulation control
stimulation+3uM IL-CHO
pNur77-Nuc

D


160
140
120
100
80
60
40
20
0
%Fold Activity
stimulation control
stimulation+100nM Comp E
pNur77-luc

E


Plasmid Control
Control beads
CompE beads
Control input
CompE input
control
CompE
IP:p300
Primer set#1

F

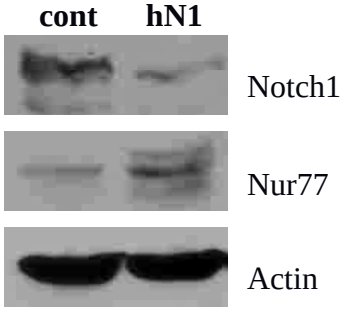

cont
hN1
Notch1
Nur77
Actin

Figure 2.15: GSI treatment or Notch1 knockdown restores stimulation induced Nur77 up-regulation in T-ALL cells

2.15A) Western blot analysis of HPB-ALL cells treated with 3uM IL-CHO or 100nM CompE and stimulated with 10 nM PMA and 500 nM Ionomycin showing Nur77 expression and actin as a internal control.

2.15B) HPB-ALL cells treated as above showing activated Notch1.

2.15C) Luciferase activity of Nur77 promoter of HPB-ALL cells treated with 3uM IL-CHO electroporated with pNur77-luc construct and stimulated for 2hr with 10nM PMA and 500 nM Ionomycin. Luciferase activity is increased with treatment with GSI.

2.15D) Luciferase activity of Nur77 promoter in HPB-ALL cells treated with 100nM Compound E electroporated with pNur77-luc construct and stimulated with 10nM PMA and 500nM Ionomycin. Luciferase activity is increased with treatment with GSI.

2.15E) Promoter binding activity of p300 in cells treated as in 5D. Treatment with GSI increases binding of endogenous p300 to the Nur77 promoter.

2.15F) Western blot analysis of Notch1 shRNA infected HPB-ALL cells showing knockdown of Notch1, Nur77 expression and actin as an internal control.

Model of Notch1 inhibition of Nur77-induced apoptosis *via* transcriptional regulation: implications for T-ALL development.

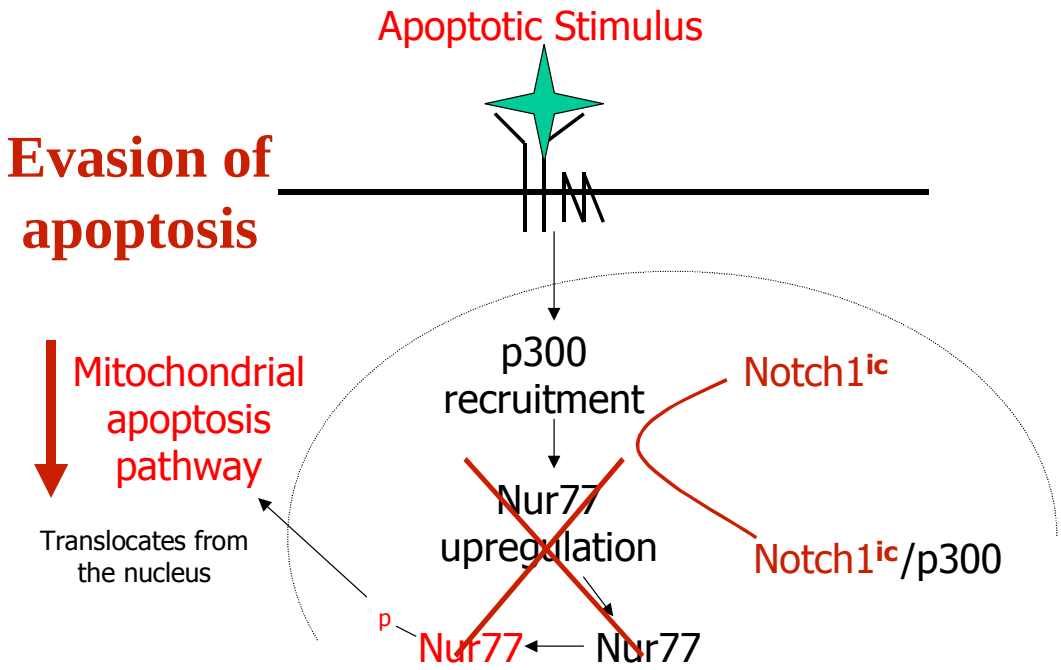


Figure 2.16: Model of Notch1 inhibition of Nur77-induced apoptosis *via* transcriptional regulation

During stimulation-induced apoptosis MEF2D that is bound to the Nur77 promoter is derepressed and recruits co-activators that include NFAT and p300, which are required for optimal transcription of Nur77. In the presence of intracellular Notch1 (Nic), Nic has the ability to directly binds with p300 decreasing its ability to be recruited to the Nur77 promoter, subsequently decreasing upregulation of Nur77. This decrease in Nur77 along with other known functions of Nic over-expression may contribute to the decrease in cell death that is seen in Nic over-expressing cells.

CHAPTER III:

IP3R REGULATION AND CALCIUM FLUX DURING NUR77 INDUCED APOPTOSIS

Introduction

The development of mature T lymphocyte takes place in a unique microenvironment known as the thymus. During the developmental period in the thymus, immature T lymphocytes, also known as thymocytes, undergo a process called negative selection. Characterized by massive apoptosis, negative selection plays a vital role in the removal of auto-reactive T-cells from the healthy mature adaptive immune repertoire. Negative selection is a dynamic process that involves multiple signaling pathways, including the regulation of intracellular calcium. It is well-established that uncontrolled calcium release plays an important role in the apoptotic process (48,50). Also well documented is the importance of the early immediate gene Nur77, a pro-apoptotic protein identified during a screening of cells undergoing negative selection. In this chapter we show that during negative selection thymocytes show a marked decrease in the amount of IP3-R3 and a subsequent increase in IP3-R1. Using DO11.10 cells with Nur77 under the control of a tet-inducible promoter we also have been able to show that this change in IP3R isoforms is Nur77 dependent.

Materials and Methods

Mice, thymocyte purification and stimulation

3-5 day old C57/B6 mice were sacrificed and thymi were removed. Thymi were mechanically homogenized in PBS and put through a 40-micron tissue culture filter to remove large amounts of tissue. Cells were then washed twice with PBS, pelleted and the supernatant was removed and the pellet was resuspended with appropriate amounts of anti-CD4 and anti-CD8 magnetic particles (BD Biosciences) and purified as per manufacturers instructions. The positive fraction from the purification was counted, resuspended in RDG complete culture media and treated with PMA/Ionomycin to simulate negative selection.

Cell culture

All cells in these experiments were previously described and treated as in Wang *et al* (45).

Immunoblotting

Gel preparation and protein transfer were done as previously described. For immunobotting primary antibodies were diluted 1:1000 or as indicated by manufacturer, with Horse Radish Peroxidase (HRP) linked secondary antibodies being diluted 1:5000. Detection was performed using Enhanced Chemiluminescence (ECL). Densitometry was performed using Image J software normalizing to internal β Actin as a control. Graphs are representative of multiple individual blots.

Antibodies

The following antibodies were used for immunoblottling: Rabbit anti-IP3R-1 (Millipore, Jaffery NH), Mouse anti-IP3R-3 (eBiosciences, San Deigo CA), Rabbit anti-GFP (Santa Cruz, Santa Cruz CA), and Mouse anti-β Actin (Sigma, St. Louis, Missouri)

Results and Discussion

Thymocytes induced to undergo negative selection show marked changes in IP3 receptor isoforms

3-5 Day old C57/B6 mice were sacrificed and thymi were removed and homogenized. The single cell suspension containing thymocytes was purified and positively selected using anti-CD4 and anti-CD8 magnetic beads. The positive fraction was resuspended in RDG cell culture media and was treated with PMA/Ionomycin to simulate negative selection. It is well-established that treatment with PMA/Ionomycin induces massive apoptosis in thymocytes and this method is acceptable as a pharmacological mimic to negative selection. Lysates from treated cells were collected over a 2h period and immunobloted for IP3R-1 and IP3R-3, which are known to be the predominant isoforms in thymocytes. To focus on the early changes in the isoforms of IP3 we performed a time course whereby we collected samples every 15 minutes for the first hour, and we then collected lysates at a two-hour time point.

Observations from the 1h time course showed little changes in the amount of IP3R-1. It is still under debate, however, some feel IP3R-1 is intimately involved in negative selection so a decrease would be very unexpected (Fig 3.1A+B) (111,112). In contrast, the amount of IP3R-3 changed over the observed time course and, in the end, showed a substantial decrease after 2 hours of treatment (Fig 3.1C+D). Interestingly, after 2h of treatment the amount of IP3R-1 showed a slight increase, as compared too untreated control lysate. These results suggest an

increased importance for IP3R-1, which is thought to be an active player in negative selection (104,105). At the same time these results simultaneously show a directed decrease in IP3R-3, which is thought to be the regulatory form of IP3R (Fig.3.1E).

Nur77 expression induces changes in IP3R similar to those observed during negative selection

A recent publication from our lab described DO11.10 cells that express Nur77 with a nuclear export sequence (Nur77-NES) under the control of a tet-responsive promoter (45). These cells can be induced to upregulate Nur77-NES as previously described in Wang et al, by removal of tet which results in an increase of GFP tagged Nur77-NES (45). Nur77-NES is consistently exported from the nucleus to the cytosol. These cells have been used to determine that Nur77-NES expression is sufficient to induce apoptosis in DO11.10 cells, highlighting the importance of Nur77 as a pro-apoptotic molecule (45). These cells provide a unique tool that can be used to examine Nur77-specific mechanisms for the induction of apoptosis, specifically its role in the cytosol. Current research on Nur77-induced apoptosis focuses on its ability to translocate from the nucleus to the cytosol where it has been shown to interact with Bcl-2 transforming it from an anti to a pro-apoptotic molecule. Nur77 is rapidly upregulated during the first 3 hours after induction of apoptosis. To determine if the changes that we had seen in IP3R during the first 2 hrs of negative selection could be under the direct influence of Nur77 we used DO11.10 tet Nur77-NES cells. Experiments performed with these cell lines suggested that 48-72h incubation is sufficient to induce apoptosis. When we

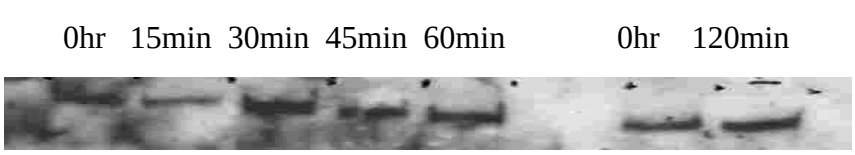
examined at the levels of IP3R-1 and IP3R-3 during a 3-day time course we observed similar trends as those seen in thymocytes, though more pronounced (Fig 3.2A). As documented, we had observed a slight increase in IP3R-1 in thymocytes induced to undergo apoptosis at 2hr. In DO11.10 over-expressing Nur77-NES resulted in distinct increases in IP3R-1 over time (Fig.3.2 A+B). Similarly, the decrease of IP3R-3 seen in thymocytes was even more noticeable in Nur77-NES over expressing DO11.10 cells (Fig.3.2 A+C). These experiments support the trends seen in thymocytes, and also suggest that the distinct changes that take place between the IP3R isoforms may be Nur77 dependent (Fig.3.2D).

Discussion

As disscussed earlier, the process of negative selection is vital for the development of a healthy, responsive adaptive immune repertoire. Nur77 has been identified as an immediate early gene that is upregulated during negative selection and is regarded as a pro-apoptotic molecule. Though the mechanism by which Nur77 initiates its pro-apoptotic program is still being disputed, it is clear that there could be multiple mechanisms that all take place simultaneously. In this report we focus on the cytosolic role of Nur77 by using a cell line that expresses Nur77 that is tagged with a nuclear export sequence. Cytosolic Nur77 has been shown to interact with members of the Bcl-2 superfamily, as well as localizing to multiple sub-cellular compartments including, but not limited to, the endoplasmic reticulum (ER) and mitochondria. Interestingly, current research on the subject of IP3R has shed new light on the interactions between the IP3R and members of the

Bcl-2 superfamily, specifically Bcl-2 (112,113). Elegant experiments by Distalhorst using a blocking peptide derived from IP3R has been able to show that blocking the interaction between IP3R and Bcl-2 is sufficient to induce apoptosis in Jurkat T-cells (112,113). Similarly, a recent report using Taxol a known chemotherapeutic drug shows that Taxol occupies a similar functional niche to Nur77 (114). These investigators were able to show that Nur77 and Taxol both bind to Bcl-2. Other reports suggest that the Taxol, beyond its anti-mitotic function, also uncouples the Bcl-2/IP3R interaction. Taken together one could foresee a model by which Nur77-localization to the cytosol could compete with IP3R for binding to Bcl-2. This competition could lead to Nur77 directed uncoupling of the protective IP3R/Bcl-2 interaction, providing yet another possible pro-apoptotic action for the cytosolic localization of Nur77. Current experiments in our lab are focused on trying to determine if this unique mechanism mediated by Nur77 is indeed plausible.

A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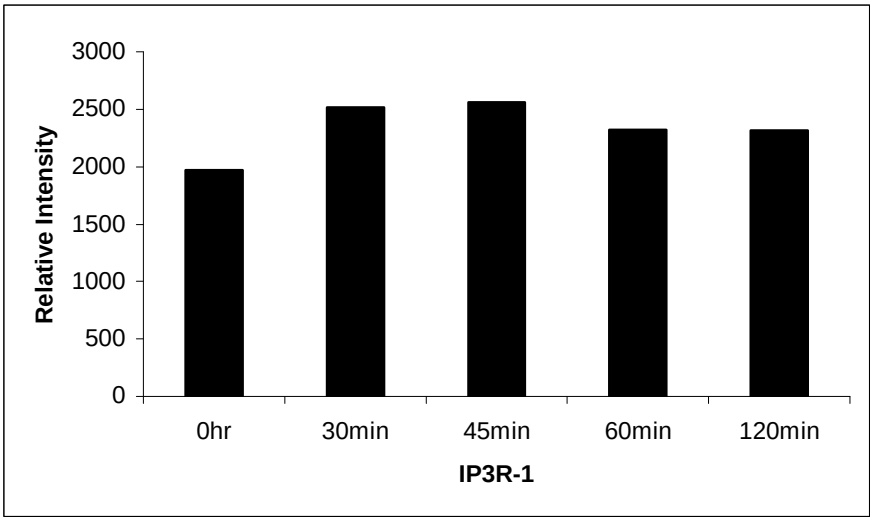
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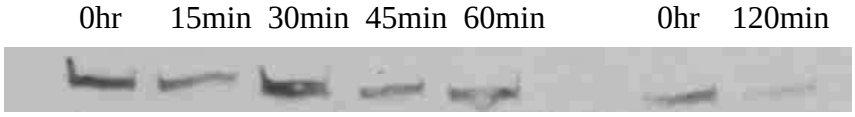
C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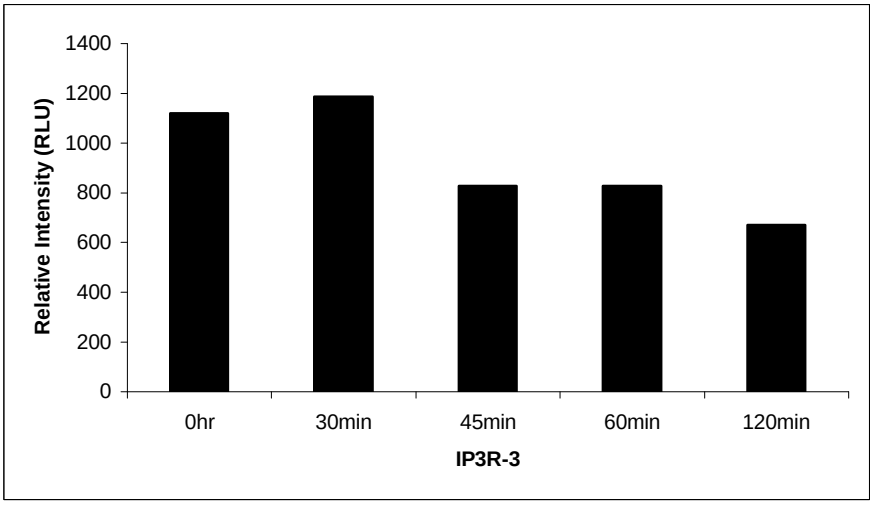
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E

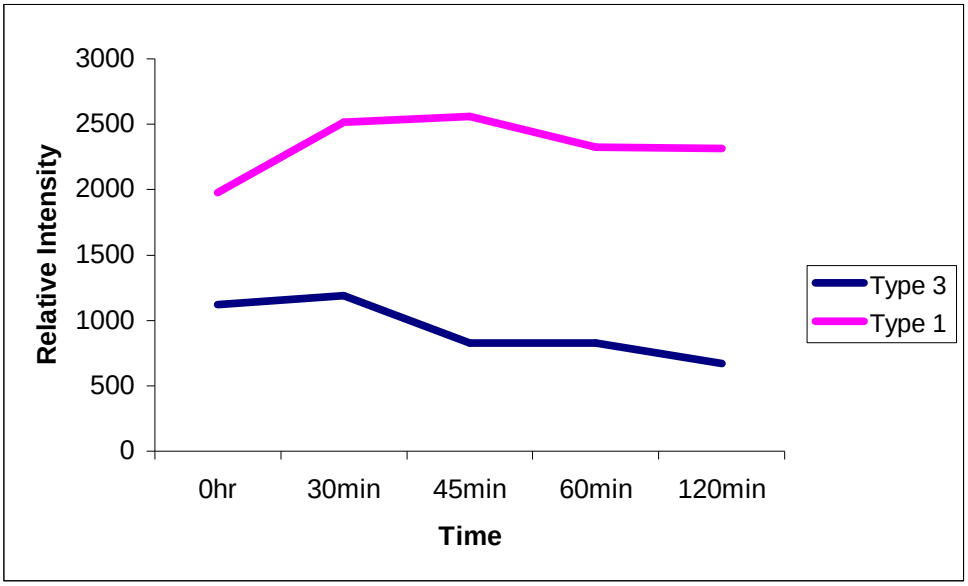


Figure 3.1: IP3R isoform levels change during PMA/ionomycin induced apoptosis in Double Positive (DP) thymocytes

20A) Western blot analysis of IP3R-1 in purified DP thymocytes treated with PMA/ionomycin for the selected time course.

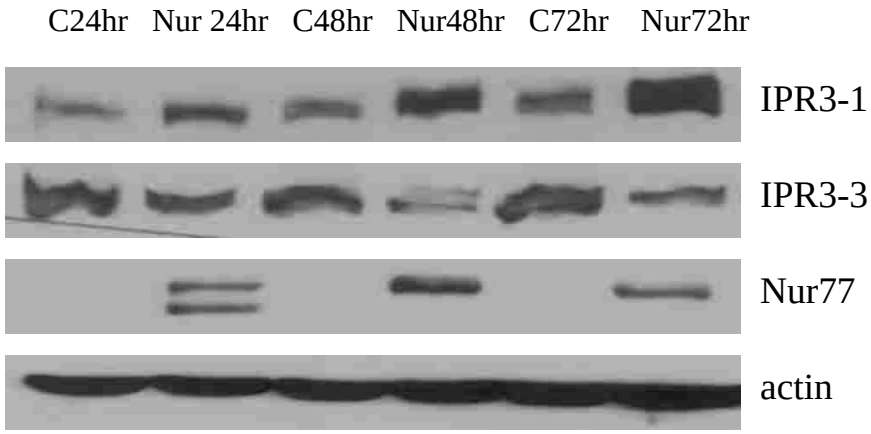
20B) Densitometric analysis of IP3R-1 western blots using Image J software, the graph is representative of multiple western blots using β-Actin as an internal control, and shown as relative intensity.

20C) Western blot analysis of IP3R-3 in purified DP thymocytes treated with PMA/Ionomycin for the selected time course.

20D) Densitometric analysis of IP3R-3 western blots using Image J software, the graph is representative of multiple western blots using β-actin as an internal control, and shown as relative intensity.

20E) Graphical representation using Microsoft Excel to show expression trends. The data points were derived from the Image J analysis to determine relative intensity. Graph shows relative intensity over the time course.

A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IPR3-1
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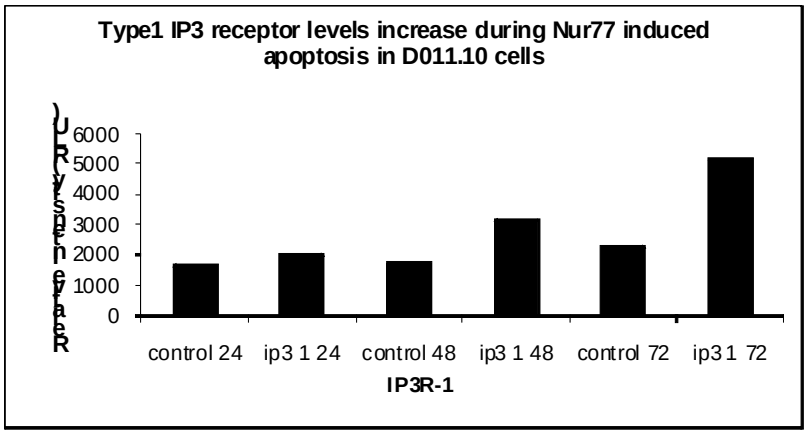
B


Type1 IP3 receptor levels increase during Nur77 induced apoptosis in D011.10 cells
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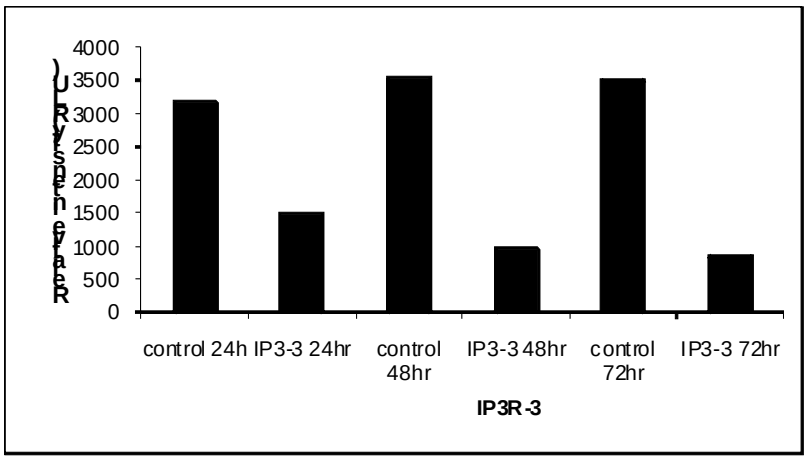
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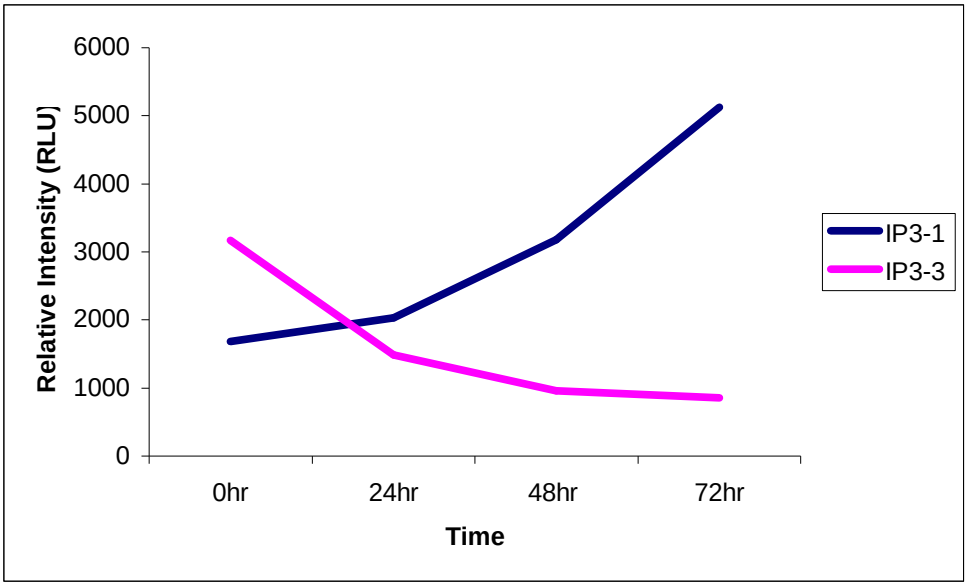

Figure 3.2: IP3R isoform levels change during Nur77 dependent apoptosis in tet responsive Nur77-NES DO11.10 cells

21A) DO11.10 cells were treated as in Wang *et al* to induce expression of GPF-tagged Nur77-NES. Whole cell lysates were collected at 24h, 48h, and 72h and subjected to immunoblotting with antibodies specific for IP3R-1 (panel 1), IP3R-3 (panel 2), Nur77-NES (panel 3), and β-actin (panel 4) as an internal control.

21B) Densitometric analysis of IP3R-1 western blots using Image J software. The graph is representative of multiple western blots using β-actin as an internal control, and shown as relative intensity.

21C) Densitometric analysis of IP3R-3 western blots using Image J software. The graph is representative of multiple western blots using β-actin as an internal control and shown as relative intensity.

21D) Graphical representation using Microsoft Excel to show expression trends over time. Numerical values for the data points were derived from the Image J analysis to determine relative intensity. Graph shows relative intensity over the time course.

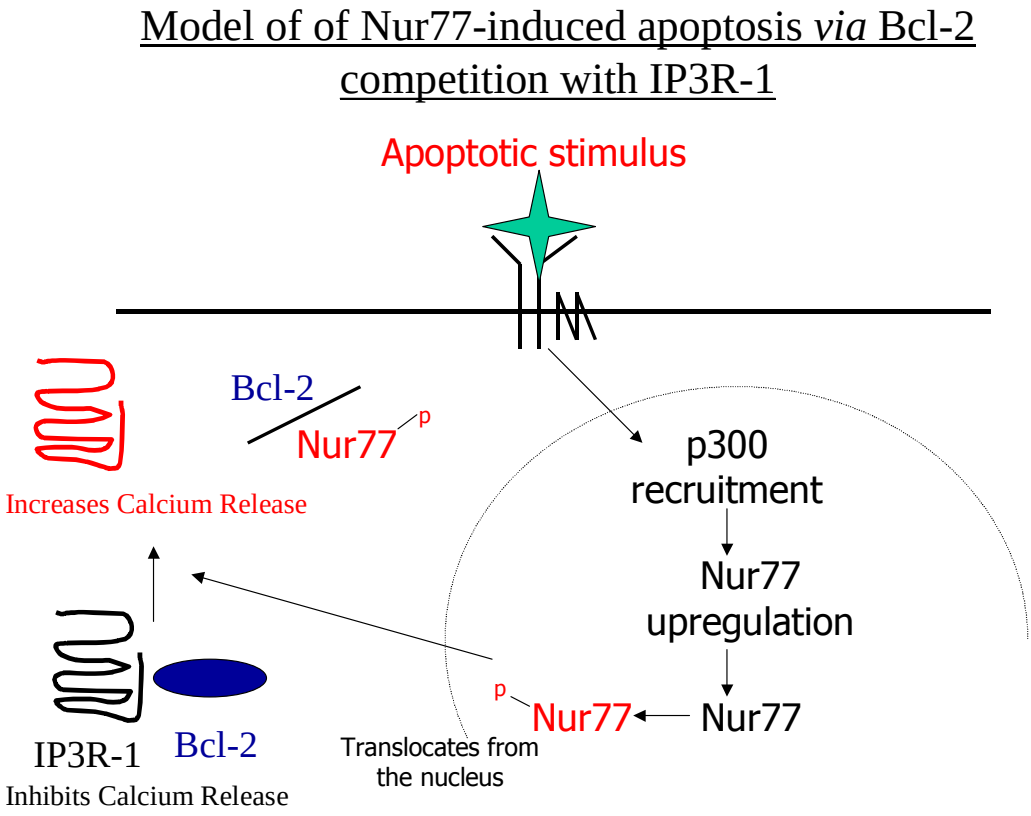

Figure 3.3: Model of Nur77 induced apoptosis *via* Bcl-2 competition with IP3 receptor

IP3 receptor, which is found in the endoplasmic reticulum, is known to regulate intracellular calcium levels, and also to interact with Bcl-2. Previous work in other labs has shown that the interaction between Bcl-2 and IP3 receptor is anti-apoptotic. By using blocking peptides that interrupted the interaction between Bcl-2 and IP3 receptor lead to an increase in internal calcium flux and increased apoptosis (88,89,103). In our model we believe that it is possible that Nur77 maybe playing a similar role to the blocking peptides by competeing with the IP3

receptor for binding to Bcl-2. Similarly we believe that by doing this Nur77 is increasing calcium flux, which would subsequently lead to increased IP3R-1 expression, as well as increased apoptosis.

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

The process of apoptosis is essential for the development of healthy complex organisms. As mentioned earlier apoptosis is a vital aspect for the development of a complete immune system. During T-cell development apoptosis plays a role in the removal of thymocytes with TCRs that do not recognize self-MHC (death by neglect), and in the removal of auto reactive T-cells (negative selection). tt has been well established that during the process of negative selection the immediate early gene, Nur77, is intimately involved in the apoptotic process. The role of Notch1 also is vital in the development of T-cells as well as other well-established functions. As important as we know Notch is to the development of T-cells, its absence from the thymocytes during the process of negative selection is made more conspicuous in light of Nic oncogenic potential. The mechanisms of Nic regulated oncogenesis are very diverse, Nic over expression has been shown to involve NFκB, c-myc, PTEN, with more targets being identified regularly. Previously published results from our lab indicate that Notch1 interacts with Nur77 and reduces its pro-apoptotic function (103). Contrary to our original interpretation of the data here we show a mechanism whereby Notch1 inhibits Nur77 and apoptosis through inhibiting Nur77 expression. We suggest that a window of opportunity is present during negative selection when Nur77 is increased and Nic is decreased. However, if active Notch1 is present this could lead to expansion of cells that are resistant to apoptosis. T-ALL leukemias frequently contain activating mutations in Nic and T-ALL cells are $CD4^+/CD8^+$ double positive. Additionally the developmental

stage at which thymocytes normally undergo negative selection leads credence to this hypothesis.

As described in detail in the second chapter of this thesis, Nur77 expression was shown to be decreased in Nic over-expressing DO11.10 cells. These decreases in Nur77 expression also lead to a decrease in cell death in Nic over-expressing cells. Notch1 is known to have multiple transcriptional regulatory functions both directly and indirectly. One potential co-factor shared between Nur77 and Notch1 is p300. Other groups have shown that Nic can negatively regulate p300 both through direct interaction but also through upregulation of Deltex1 (109). Nur77 upregulation in response to apoptotic stimuli has been shown to be dependent on p300, NFAT, and MEF2D (48,50). Early experiments focus on MEF2D as the possible shared factor between Nur77 and Nic, however data were inconclusive. Similar to other experiments with MEF2D to determine this possible regulation we assayed Nur77 promoter activity in the presence of p300 and Nic, and promoter binding under the same conditions. In the presence of Nic there was a marked suppression of Nur77 promoter activity with a subsequent loss of p300 binding. More important than identification of this mechanism is our ability to provide information using GSI and shRNA for hNotch1 in T-ALL cell lines that shows that our proposed mechanism is possible in a model of the disease.

Throughout the development of this model we believe that it would have been beneficial to show that this mechanism is also viable *in vivo* using a mouse model of the disease. Experiments using the TOP-NOTCH mice model were met with problems with the mice themselves, and no results were ever recorded (101). One avenue that could be

interesting is to determine the validity of this mechanism using primary patient samples from a cooperative hospital. A simple set of experiments using GSI treatment similar to what was done with our T-ALL cell line would highlight the complexity of the individual samples. The mutations that are present in the T-ALL cells have been documented so one can realistically gauge the effectiveness of a potential treatment with GSI if the mutation is in the HD domain. If the mutation leading to the over expressed Nic was in the PEST domain or part of a genetic translocation to an active promoter, then GSI would have no effect. The determination of the mutation in a primary sample would have to be quick so as not to expose the cells to prolonged culture conditions, so they would still be considered primary samples. Overall, we have provided compelling information that a previously documented mechanism of promoter regulation by Nic could be involved in the development of T-cell Acute Lymphoblastic Leukemia *via* the regulation of the Nur77.

As part of a cooperative effort between our lab and the Fissore lab, we also decided to look at changes in the IP3R during the process of negative selection. We know that the process of negative selection in thymocyte is a process that takes place over a significant amount of time. We were specifically interested in the early events of negative selection that could be causing changes in the status of IP3R. To do this, a time course was performed using thymocytes stimulated with PMA/Ionomycin, to induce cell death, and western blots were performed to access IP3R levels and changes in molecular weight. It is well document that IP3R undergos a cleavage event during apoptosis, which leads to a loss in regulation and increased calcium flux. Other groups have shown that this

increased flux is important to sustain the apoptotic program. Also of interest at this time was the possible role of a known pro-apoptotic molecule Nur77. Recent publications have shown that the IP3R interactions with members of the Bcl-2 family are an important regulatory mechanism during activation-induced apoptosis (111,112,113). Observations by others suggest an interaction of Nur77 with Bcl-2, which has been shown to be part of the pro-apoptotic mechanism of Nur77 (114). Though not a complete story, results from our experiments suggest a role for Nur77 in the unique changes that are observed in IP3R isoforms during PMA/Ionomycin induced apoptosis.

Double positive thymocytes that were stimulated with PMA/Ionomycin over the course of 2hrs showed distinct changes in the amount of IP3R-3, specifically at the 2hr time point. We also observed a very slight increase in the amount of IP3R-1 during this same time course. To determine whether Nur77 played any role in these changes, tet-Nur77-NES DO11.10 cells were induced to express Nur77-NES and lysates were collected over the course of 3 days. Experiments to determine the optimal apoptotic time points in these cells had been determine previously. Western blot analysis of lysates from these cells showed more pronounced changes in the IP3R isoforms, providing information about the importance of Nur77 in these changes. As seen in thymocytes, Nur77-NES expression in these cells lead to an increase of IP3R-1 over the time course, while showing a substantial decrease in IP3R-3 over the same period. Previously published results from our lab have show that at 72 hours after induction apoptosis is at its peak in these cells; this coincides with the highest amount of IP3R-1expression and the concomitant loss of IP3R-3. These results suggest the involvement of Nur77 in a

wholly-new mechanism, which involves the regulation of IP3R isoforms during the initiation of activation-induced apoptosis.

Future experiments on this project could be focused on the possible mechanism by which Nur77 is eliciting the changes in IP3R isoforms during apoptosis. As mentioned earlier, the interaction of Nur77 with Bcl-2 seems to be an obvious possible event during these changes. It has been established in other model that increased in cytosolic calcium and activation of specific calcium-inducible factors leads to increased expression in IP3R-1. Information provided in the literature has shown that IP3R-1 and Bcl-2 interact, and that this interaction keeps IP3R-1 in a conformationally closed state (112,113). In the same report, the authors showed that a blocking peptide that inhibited this interaction led to the opening of IP3R-1 and a subsequent increase in calcium (111,112). We could speculate that Nur77, which is a known interacting partner of Bcl-2, could be playing a similar role in interrupting the interaction between IP3R-1 and Bcl-2. Though this would account for the changes in IP3R-1, it is still unclear how this mechanism would cause the changes seen in IP3R-3. Additionally, although its thought that IP3R-3 is a more regulatory form of IP3R, it remains unclear how the complexities of this mechanism could be causing such drastic changes so fast without using proteosomal degradation. We have preliminary data that suggest the proteosome is indeed not utilized during this process, but experiments must be repeated toconfirm these results.

During the actual commission of all the experiments done for this thesis it was unclear how these two projects would intersect. After compiling the data however it became very clear to me that as Nic decreases the amount of Nur77, this could also lead to an

inhibition of the changes that we see in IP3R isoforms. It would be interesting to over-express Nic in the tet-Nur77-NES cells and determine if Nic does indeed effect the changes that we see in the presence of Nur77-NES alone. Taken together we provide compelling data supporting a mechanism by which Nic can inhibit Nur77-upregulation and at the same time show that there can be an immediate downstream consequence that could alter apoptosis *via* Nur77.

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