

THE ROLE OF THE CELL SURFACE PROTEASE RECEPTOR S100A10 IN ACUTE  
PROMYELOCYTIC LEUKEMIA (APL) AND ITS REGULATION BY RETINOIC  
ACID THERAPY

by

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I dedicate this thesis in the memory of Mary Suzanne (Mary Sue) Waisman,  
my friends who were taken far too soon due to leukemia, Christopher 'Kit'  
Colwell and Marcia Fiolek, and my Grandfathers, Alvin Reid and Allan 'Bull'  
Holloway.

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## **ABSTRACT**

Acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia, is characterized by expression of the promyelocytic leukemia-retinoic acid receptor oncoprotein, PML/RAR $\alpha$ , which blocks granulocyte differentiation. Patients with APL present fatal hemorrhagic complications from excessive generation of the fibrinolytic protease plasmin and the procoagulant protein, tissue factor (TF). We previously demonstrated that a cell surface regulator of plasmin generation, S100A10 (p11) is upregulated in APL promyelocytes. Treatment of APL promyelocytes with all-trans retinoic acid (ATRA) mitigates the hemorrhagic disorder concomitant with promoting the degradation of PML/RAR $\alpha$  and p11. Here, we show PML/RAR $\alpha$  up-regulates p11 transcript and protein levels concomitant with increased plasmin activity. Depletion of p11 from the PML/RAR $\alpha$  expressing APL promyelocyte cell line, NB4, reduced plasmin activity, but not TF activity. ATRA treatment of NB4 cells reduced p11 transcripts and promoted its ubiquitin-independent proteasomal degradation. Unexpectedly, the ATRA-dependent loss of p11 increased the plasmin activity and decreased the TF activity of the NB4 cells. However, ATRA treatment of MCF-7 cells also blocked p11 transcription and protein levels suggesting that ATRA can regulate p11 levels independent of PML/RAR $\alpha$ . These studies highlight the complex regulation of p11, demonstrate that changes in p11 do not always correspond with decreases in plasmin generation, and challenge the hypothesis that p11 is regulated by ubiquitin-mediated proteasomal degradation.



## **LIST OF ABBREVIATIONS AND SYMBOLS USED**

$\alpha_2$ -AP	alpha 2-anti plasmin
AII <sub>t</sub>	annexin A2 heterotetramer
AML	acute myeloid leukemia
AP-1	activator protein-1 transcription factor
APL	acute promyelocytic leukemia
APS	ammonium persulfate
ATRA	all-trans retinoic acid receptor
BSA	bovine serum albumin
dH <sub>2</sub> O	distilled water
DLC-1	deleted in liver cancer 1
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMT	epithelial–mesenchymal transition
FACS	fluorescent activated cell sorting
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hank's balanced salt solution

HPRT1	Hypoxanthine Phosphoribosyltransferase 1
I $\kappa$ B	inhibitor of kappa B
IKK	I $\kappa$ B kinase
KO	knockout
MEM	minimum essential medium
NF $\kappa$ B	nuclear factor kappa B
NLS	nuclear localization signal
NT	non-transgenic
PAGE	polyacrylimide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pg	plasminogen
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
Pm	plasmin
PMA	phorbol 12-myristate 13-acetate
PML	promyelocytic leukemia
PML/RAR $\alpha$	promyelocytic leukemia-retinoic acid receptor-alpha protein
PMSF	phenylmethylsulfonyl fluoride
qPCR	quantitative polymerase chain reaction
RAR $\alpha$	retinoic acid receptor alpha
RPMI	Roswell Park Memorial Institute media

RTK	receptor tyrosine kinase
RXR	retinoic X receptor
SDS	sodium dodecyl sulfate
STAT	signal transducer and activator of transcription
Suc-LLVY-AMC	Suc-Leu-Leu-Val-Tyr-AMC
TAE	tris-acetate-EDTA
TBST	tris-buffered saline and tween 20
TEMED	N,N,N',N'-Tetramethylethane-1,2-diamine
TF	Tissue Factor
TG	thioglycollate
tPA	tissue plasminogen activator
Ubiquitin-KO	ubiquitin-all lysines mutated to arginine
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
WB	Western blotting
WT	wild type

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## **CHAPTER ONE: INTRODUCTION**

### **1.1. Cell Surface Plasmin Activity**

#### **1.1.1. Introduction**

Proteases are essential for the breakdown of the proteins of blood clots formed by fibrin and the extracellular matrix (ECM), which facilitates the migration of immune cells and the metastatic spread of cancer cells. Serine proteases and other proteases perform these actions, which are regulated by numerous activators, inhibitors and cellular receptors. Plasmin is a one of the predominant serine proteases with a broad spectrum of substrates in the ECM and blood. Its proteolytic function is central to several key physiological and pathological processes that including inflammation, metastasis, and fibrinolysis.

#### **1.1.2. Extracellular Proteases: Regulators of Tissue Remodelling and Blood Fluidity**

Extracellular proteolysis is an essential mechanism that remodels the extracellular environment by degrading the ECM. This permits the migration of cells not only in numerous essential biological processes as wound healing, inflammation, and embryogenesis, but also in pathological processes such as cancer cell invasion and metastasis. Extracellular proteolysis also plays a critical role in maintaining blood fluidity through the digestion of fibrin clots, a process called fibrinolysis (Reviewed in <sup>1,2</sup>). Enzymes called proteases mediate proteolysis and consist of a heterogeneous group of endopetidases, which cleave non-terminal amino acids of proteins, and exopeptidases that cleave terminal amino acids of proteins. Proteases are classified by the residue of their active site and for the mechanism of proteolytic cleavage. Extracellular proteases are

endopeptidases that include metalloproteinases, serine proteases and cysteine proteases (Reviewed in <sup>1</sup>). The metalloproteinases and serine proteases are the principal enzymes in tissue remodeling and fibrinolysis because they function optimally at a neutral pH, whereas cysteine proteases function optimally at an acidic pH in locations like lysosomes.

The metalloproteinases are a family of calcium-dependent, zinc-containing endopeptidases that typically degrade ECM proteins and activate/de-activate various ligands. They are characterized by the HExxH (histidine-glutamic acid-x-x-histidine, where x is any residue) or HExxHxxGxxH zinc-binding motifs located in the catalytic domain (Reviewed in <sup>1,2</sup>). MMP protease activity is activated by zinc ions binding to the His-triad of the HExxHxxGxxH zinc-binding motifs. Although several mechanisms of how zinc-bound MMPs cleave substrates have been proposed, it is generally believed that Zn<sup>2+</sup> and the glycine amino acid of HExxHxxGxxH coordinate to break the peptide bond of proteins targeted by MMPs<sup>3,4</sup>. The metalloproteinases consist of several groups, but the main groups are matrix metalloproteinases (MMP) and adamalysins (ADAMs). The MMPs have 24 family members; and the majority are grouped based on their substrates: matrilysins, collagenases, gelatinases, stromelysins<sup>5</sup>. The MMPs generally have three domains: a catalytic metalloproteinase domain, a variable hinge region, and a hemopexin (Hpx) domain. Members of the family of MMPs degrade several key components of the ECM. For example, MMP2 and MMP9 degrades type IV collagens, elastin, gelatin and fibronectin, and MMP3 degrades collagens, gelatin, and plasminogen<sup>6</sup>. ADAMs are a group of 30 members and are identified based on their two key domains: the cells surface adhesion disintegrin domain and the carboxy-terminal metalloproteinase domain<sup>7</sup>.

ADAMs consist of transmembrane and secreted proteins that were initially shown to play key roles in fertility by facilitating the association between the sperm and egg<sup>8</sup>. The latter function is believed to involve interactions between the disintegrin domain of ADAMs on the sperm and the integrins expressed on the egg<sup>8,9</sup>. Like the MMPs, ADAMs also promote tumour invasion and metastasis of various cancers (i.e. breast<sup>10,11</sup>, colon<sup>12</sup>, brain<sup>13</sup>, etc) through degradation of the ECM.

Serine proteases are named based on the nucleophilic serine residue contained within their active site, which is mediated by a catalytic triad of Asp–His–Ser<sup>14,15</sup>. The serine proteases cleave peptide bonds at carbonyl groups. Serine proteases are a heterogeneous family of enzymes mainly associated with the vasculature and contribute to cellular invasiveness. Key regulators of coagulation and fibrinolysis are typically serine proteases that circulate in the blood. The coagulation cascade involves a series of serine protease-mediated cleavage of substrates by the extrinsic and intrinsic pathways (Figure 1; Reviewed in <sup>16,17</sup>). Cleavage of the substrates will induce their serine protease activity and cleave the next substrate in the coagulation cascade. Initiation of the extrinsic coagulation pathway begins with the binding of the serine protease factor VII (FVII) to tissue factor (TF), which then allows the FVII-TF complex to bind and subsequently activate factor X (FX). Initiation of the intrinsic coagulation pathway occurs when the serine protease, factor XII (FXII), is activated when associated with collagen, prekallikrein (PK) and high-molecular-weight kininogen (HK). This cleaves PK to produce kallikrein, which activates FXII. Activated FXII activates factor XI (FXI), which then activates factor IX (FIX). FIX associates with factor VIII to then proteolyze and activate FX. Activated FX (FXa) is released and converts prothrombin to the serine

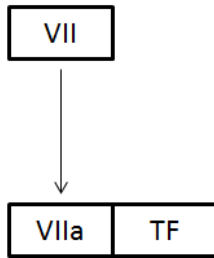


protease thrombin. Thrombin then stimulates fibrin clot formation through cleavage of its precursor fibrinogen to fibrin monomers (reviewed in <sup>18</sup>). Fibrinolysis requires the serine-protease-dependent cleavage of plasminogen, which becomes an active serine protease called plasmin. Plasmin cleaves the peptide bond between lysine and arginine residues of substrate proteins. Plasmin proteolyzes a broad range of substrates such as fibrin<sup>19</sup>, fibronectin<sup>20</sup>, von Willebrand factor<sup>20</sup>, thrombospondin<sup>20,21</sup>, extracellular matrix proteins (e.g. laminin<sup>22</sup>), and MMP precursors<sup>23</sup>, which further degrade ECM proteins. The main physiological functions of plasmin are regulating fibrinolysis and cellular invasion. Irregular activity of plasmin has been attributed to pathological roles in cancer cell metastasis as well as thrombosis, stroke, and bleeding disorders.

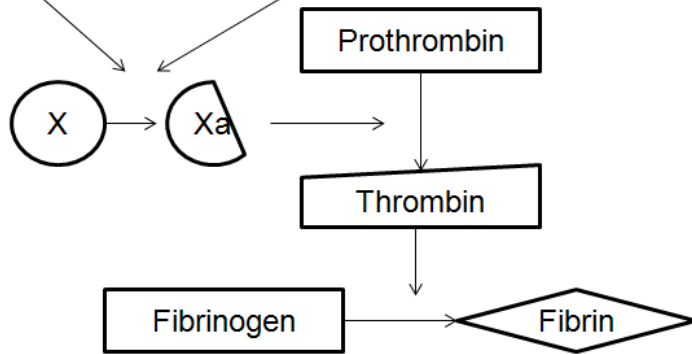
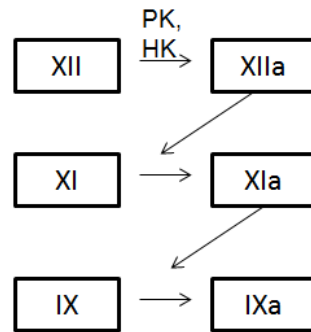
**Figure 1: The Coagulation Pathways.**

The main coagulation pathways are A) the extrinsic pathway and B) the intrinsic pathway. In the extrinsic pathway, tissue factor (TF) on the cell surface associates with factor VII (VII) following blood vessel damage, and the TF-VII complex activates factor X ( $X \rightarrow X_a$ ). In the intrinsic pathway, a series of activation of serine proteases occurs. First, factor XII (XII), prekallikrein (PK) and high-molecular-weight kininogen (HK) will form complexes on collagen. Prekallikrein is cleaved to kallikrein and factor XII becomes activated (XIIa). Factor XIIa activates factor XI ( $XI \rightarrow XI_a$ ), and then factor XI activates factor IX ( $IX \rightarrow IX_a$ ). Factor IXa then activates factor X. Both extrinsic and intrinsic pathways lead to conversion of prothrombin to thrombin by factor X. Thrombin cleaves fibrinogen to produce fibrin that will form fibrin clots to attenuate bleeding.

Extrinsic Coagulation Pathway



Intrinsic Coagulation Pathway



### 1.1.3. Regulation of Plasminogen Activation

Plasmin is produced from its precursor plasminogen, a 92-kDa single-chain zymogen that is secreted from the liver into the blood at a concentration of 2  $\mu$ M and a half-life of 2-days<sup>24</sup>. Plasminogen is initially produced in the form of amino-terminal glutamic acid plasminogen (glu-plasminogen) that consists of seven domains<sup>2,25</sup>: 1) the amino-terminal pan-apple-nematode (PAN) domain, 2-6) five kringle domains (K1-K5) and 7) a serine protease domain. In its inactive and less activatable form, glu-plasminogen maintains a closed conformation with the PAN domain interacting with the kringle domains. The kringle domains of plasminogen are responsible for regulating protein-protein interactions where the plasminogen can bind to fibrinogen as well as various cell surface plasminogen receptors at their carboxy-terminal lysines. The K1 and K4 kringle domains of plasminogen have the strongest affinity for binding to carboxy-terminal lysines, and K3 lacks a lysine-binding site<sup>2,25-27</sup>. When bound to the cells, glu-plasminogen can be cleaved by plasmin to remove the PAN domain and produce the more active form of plasminogen, lys<sup>77</sup>-plasminogen<sup>2,25</sup>. The conversion of glu-plasminogen to lys-plasminogen causes an increase in the rate of plasmin generation by plasminogen activators<sup>2,25,28</sup>.

To produce plasmin, plasminogen is cleaved between Arg561 and Val562 by plasminogen activators<sup>2</sup>. Tissue plasminogen activator (tPA) is one of the two main plasminogen activators produced mainly from endothelial cells, oocytes, keratinocytes, microglial cells and neurons, and is predominantly involved in vascular fibrinolysis<sup>2,29</sup>. tPA is produced as a 72-kDa single chain protein (scTPA) that is cleaved at Arg275-Ile276 by plasmin or kallikrein to two-chain form (tcTPA)<sup>30-32</sup>, resulting in a 15-fold

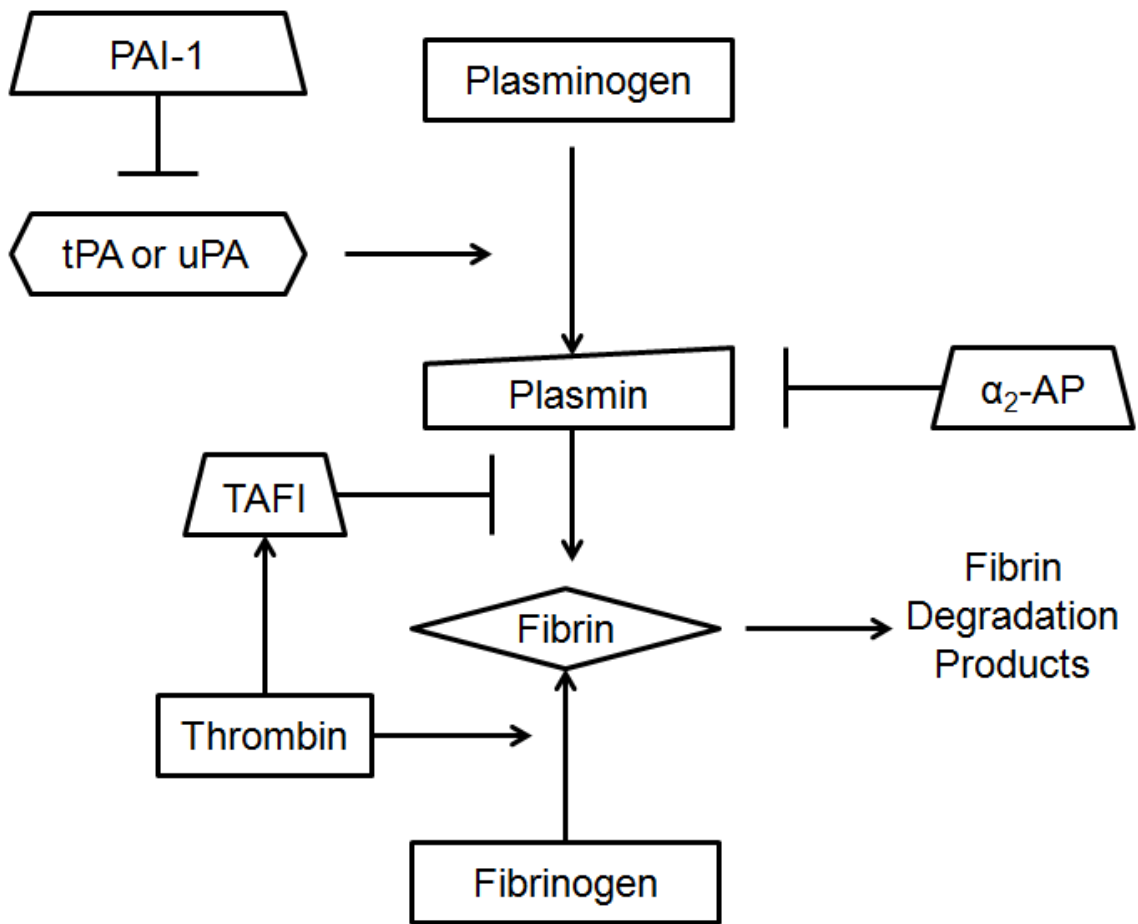
increase in tPA activity<sup>33</sup> (Figure 2). However, sctPA bound to fibrin enhances tPA activity to similar levels as tctPA<sup>34</sup>. Similar to plasminogen, tPA contains kringle domains (K1 and K2) that facilitates its binding to plasminogen activator receptors located on the cells surface, which consequently mediates the co-localization of plasminogen with plasminogen activators. Urokinase plasminogen activator (uPA), the second main plasminogen activator is produced as a single-chain glycoprotein by leukocytes, macrophages, fibroblasts and tumour cells<sup>19,35</sup>, and is a major contributor of plasmin-dependent ECM degradation and metastasis<sup>36-38</sup>. The activation of uPA is regulated by the uPA receptor (uPAR) on the cell surface, which binds the uPA precursor, pro-uPA, and localizes it to the cell surface<sup>39</sup>. Plasmin or kallikrein causes the cleavage of single chain pro-uPA at Lys158-Ile159 to produce disulphide-linked two-chain uPA, which has greater activity than pro-uPA<sup>19</sup>. Once active, the catalytic triad of uPA (consisting of His204, Asp255, and Ser356)<sup>40</sup> is responsible for the cleavage plasminogen to plasmin. The cleavage of plasminogen by uPA or tPA produces a two-chain form of plasmin that is composed of the heavy A chain (561 amino acids, 60 kDa) and light B chain (230 amino acids, 25 kDa). These chains are held together by two disulfide bonds and stabilized by a salt bridge at the amino-terminal Val-562 and Asp-740<sup>2,25</sup>. The activity of plasmin depends on the catalytic triad consisting of His603, Asp646, and Ser741 in light chain B<sup>41,42</sup>. Plasmin can circulate in the blood and promote fibrinolysis, (Reviewed in <sup>2,25</sup>) or degrade the ECM within tissues.

Fibrinolysis is a tightly regulated process with multiple checkpoints at various steps. Plasminogen activator inhibitors-1 and -2 (PAI-1, PAI-2) block uPA and tPA from cleaving plasminogen. PAI-1/-2 associates with uPA or tPA non-covalently, causing the

cleavage of the Arg346-Met347 bond in PAI<sup>2,43</sup>. Arg346 of PAI then binds covalently to Ser356 of uPA or Ser478 of tPA. Plasmin is inhibited directly through  $\alpha_2$ -antiplasmin ( $\alpha_2$ -AP). Initially,  $\alpha_2$ -AP associates with plasmin non-covalently and leads to the cleavage of the Arg346-Met347 bond in  $\alpha_2$ -AP<sup>2,44,45</sup>. Arg346 of  $\alpha_2$ -AP then binds covalently to Ser741 in the active site of plasmin, resulting in the inhibition of plasmin-dependent proteolysis<sup>2,45</sup>. Thrombin activatable fibrinolysis inhibitor (TAFI) is a single-chain, zinc-dependent carboxypeptidase activated by cleavage at Arg92 by thrombin, which is produced from activation of the coagulation cascade<sup>46</sup>. TAFI then cleaves and removes the carboxy-terminal lysines of fibrin and plasminogen receptors and prevents the binding of plasminogen, uPA, and tPA. Hence, TAFI acts to attenuate the activation of plasminogen by various regulators.

**Figure 2: Regulation of Plasmin Activation.**

Plasminogen is cleaved by the plasminogen activators: tissue and urokinase plasminogen activator (tPA and uPA, respectively). The cleavage of plasminogen by tPA or uPA results in the production of plasmin. In the blood, thrombin produces fibrin clots, and plasmin destroys fibrin clots through proteolysis of fibrin. Plasminogen activator inhibitors (PAI-1 or PAI-2) can bind to tPA or uPA and inhibit them from cleaving plasminogen. Plasmin can be inhibited by  $\alpha_2$ -antiplasmin ( $\alpha_2$ -AP) where  $\alpha_2$ -AP prevents plasmin activity by binding to its active site. Thrombin can activate thrombin activatable fibrinolysis inhibitor (TAFI), which prevents plasminogen activation by removing carboxy-terminal lysine from fibrin and plasminogen receptors that bind and co-localize plasminogen with its activators, tPA and uPA.





#### 1.1.4. Cell Surface Plasminogen Receptors

Although plasminogen activators convert plasminogen to plasmin, plasminogen activation is more efficiently activated by plasminogen receptors at the cell surface<sup>2</sup> that can co-localize plasminogen with its activators. Plasminogen receptors (Table 1) consist of a heterogeneous group of cell surface proteins or non-proteins (i.e. gangliosides<sup>47</sup>) that immobilize plasminogen and significantly increase the proteolytic conversion of plasminogen to plasmin. Lysine-binding sites contained in the K1 and K5 domains of plasminogen<sup>28</sup> are the sites that bind to cell surface plasminogen receptors typically at carboxy-terminal lysines<sup>48</sup>. The K1 domain of the closed conformation plasminogen mediates the binding to its substrates such as plasminogen receptors<sup>49</sup>. In contrast, the K5 domain of the closed conformation plasminogen is responsible for the initiation of the conformational change leading to the open conformation of plasminogen. The major plasminogen receptors include  $\alpha$ -enolase, histone H2B, Plg-R<sub>KT</sub>, amphoterin (also known as high-mobility group box 1; HMGB1), annexin 2 (p36)-S100A10 (p11) heterotetramer (AIIIt; described in section 2.0) and S100A4<sup>25</sup>. The plasminogen receptor  $\alpha$ -enolase is a 45 kDa metalloenzyme expressed in most cell types, and is mainly expressed in hematopoietic, endothelial and neuronal cells<sup>50</sup>. Histone H2B is a 17 kDa protein that can be found in the nucleus with other histones that regulate transcription, but it is also an extracellular membrane-associated protein where it acts as a plasminogen receptor<sup>25,51</sup>. Cell surface histone H2B is found mainly in monocytes, macrophages, activated lymphocytes, and lung carcinoma cells<sup>25,52</sup>. Plg-R<sub>KT</sub> is a 17 kDa integral membrane protein and is expressed in a broad number of tissues<sup>53</sup>. Plg-R<sub>KT</sub> also co-localizes with uPAR and its plasminogen binding sites are also capable of binding tPA<sup>25,53</sup>. HMGB1 is a

30 kDa protein localized to the nucleus where it acts as a DNA-binding protein that regulates transcription by stabilizing nucleosome formation and also promoting transcription<sup>54,55</sup>. It is also found at the cell surface where it interacts with toll-like receptor 4 (TLR4) to modulate inflammatory responses<sup>55</sup> and can bind plasminogen<sup>56</sup>. Like its relative, p11, S100A4 can also form a heterotetramer complex with p36, where it can bind plasminogen at the cell surface.

**Table 1: The Major Cell Surface Plasminogen Receptors.**

<b>Plg receptors</b>	<b>C-terminal lysine</b>	<b>Major presence in cell types</b>
Actin <sup>57</sup>	Absent	Endothelial, carcinoma
$\alpha$ -enolase <sup>58</sup>	Present	Monocytes, neutrophils, carcinoma, lymphoid, myoblast neurons
Cytokeratin 8	Present	Epithelial cells, breast carcinoma
Histone H2B <sup>51,52</sup>	Present	Neutrophils, monocytoïd cells, endothelial cells
HMGB1 <sup>56</sup>	Present	Various
Plg-R <sub>KT</sub> <sup>53</sup>	Present	Monocyte, macrophages, neuronal cells
S100A4 <sup>59</sup>	Present	Various
S100A10 (p11) <sup>60</sup>	Present	Various

### **1.1.5. Role of Plasmin in Fibrinolysis**

Plasminogen circulating in the blood is activated by binding to blood clots. Once activated, plasmin mediates fibrinolysis by proteolyzing the fibrin clot produced by the coagulation cascade (Reviewed in <sup>2</sup>). As described in section 1.1.1, the coagulation cascade produces fibrin through cleavage of its substrate, fibrinogen, which also circulates in blood. Fibrinogen cleavage is followed by the cross-linking of fibrin with platelets and other proteins, which leads to the obstruction of bleeding. Because fibrin possesses a carboxy-terminal lysine, it is able to bind plasminogen. Fibrin binds K1 and K4 of plasminogen as well as K2 of tPA, but does not bind uPA<sup>2,37</sup>. By co-localizing plasminogen and tPA, fibrin stimulates plasmin activity, which consequently promotes the cleavage of fibrin<sup>61</sup>. The products of plasmin-cleaved fibrin also contain carboxy-terminal lysines that further enhance plasmin production and the clearance of the fibrin clot.

### **1.1.6. Role of Plasmin in Cellular Migration and Metastasis**

One process important for cellular migration is the plasmin-dependent degradation of the extracellular and basement membranes<sup>62,63</sup>. This allows immune cells to infiltrate inflammation or tumour sites, and invasive cancer cells to migrate into the vasculature and new tissues. Although plasmin has a major role in fibrinolysis, it contributes to the degradation of the ECM to facilitate cancer cell migration<sup>64,65</sup>. Tumour development and progression, a process called oncogenesis, depends on plasmin to proteolyze the ECM and basement membrane to facilitate the growth of the tumour. Plasmin-dependent degradation of the ECM and basement membrane is also essential for the metastatic spread of cancer cells by promoting the escape of tumour cells into the

vasculature<sup>65</sup>. Circulating tumour cells also use plasmin to metastasize to other tissues and form secondary tumours. Szende *et al*<sup>66</sup> examined the effect of plasmin inhibition on tumour growth using the novel plasmin inhibitor YO-2. They showed that plasmin inhibition in a mouse xenograft model using the transplantation of several human cells lines including colon cancer (HT-29), melanoma (HT-18) and lymphoma (HT-58) reduced tumour growth of all xenografts and attenuated lung metastasis of the melanoma xenograft mice. The expression of uPA and uPAR are typically high in several cancers including neuroblastoma<sup>67</sup>, melanoma<sup>68</sup>, and breast<sup>69</sup>. Several studies have identified that high levels of uPA is associated with high metastasis risk in breast cancer patients<sup>65,69,70</sup>.

## **1.2. The S100A10-Annexin A2 Heterotetramer**

### **1.2.1. Annexin A2 (p36), S100A10 (p11) and AIIIt Heterotetramer Structure**

In most cell types, one of the significant contributors to the total amount of plasmin generated is the annexin A2 (p36)-S100A10 (p11) heterotetramer complex, also known as AIIIt<sup>60,71</sup> (Reviewed in <sup>72</sup>). The p11 subunit of AIIIt is present as an 11 kDa protein in endothelial cells, macrophages, and most cancer cells and is highly conserved among vertebrates<sup>73</sup>. P11 is a member of the S100 family of calcium-binding, dimeric EF hand-type proteins that range in size of 9-13 kDa. The S100 family consists of 24 genes, in which 19 of the S100 genes are located on chromosome 1q21 (S100A1-S100A19) and four genes located elsewhere (S100A11P, S100B, S100G, S100P, and S100Z)<sup>73-75</sup>. Most members of the S100 proteins contain two EF-hand motifs and each motif consists of two  $\alpha$ -helices linked with a calcium-binding loop located at the amino-terminus and carboxy-

terminus of the S100 protein<sup>74</sup>. In all S100 proteins, the amino-terminal EF-hand motif (referred to as the S100-specific or pseudo EF domain) consists of a 14-amino acid calcium-binding loop, which binds calcium using the backbone carbonyl groups and one carboxylate side-group of glutamic acid. P11 differs from other S100 proteins in that it is unable to bind calcium<sup>73</sup>. The p11 protein consists of four  $\alpha$ -helical domains that vary in length (HI (Q3-A19), HII (K27-K36), HIII (A50-L58) and HIV (F68-H89)) with a loop region separating HI-II (L1 (A19-L30)) and HIII-IV (L2(D59-S70)), and a hinge region between HII-III (HR1 (P39-N44))<sup>73</sup>. The first EF hand of p11 is composed of H1-L1-HII and the second is composed of HIII-L2-HIV. The S100 proteins typically form homodimers through interactions between helices I and IV, where the S100 dimers are in an anti-parallel orientation. Upon calcium binding, there is a conformational change resulting in a reorientation of HIII, and some minor changes in the amino-terminal EF-hand and HIV. This reorientation shifts HIII to be positioned nearly perpendicular to HIV, causing the surface exposure of five hydrophobic residues on HIV, two hydrophobic residues on HI and three hydrophobic residues in the HR1 regions. P11 contains three deletions within L1 of the first EF-hand and two substitutions of glutamic acid and asparagine residues in the second EF-hand that attenuates calcium binding to p11. The substitutions present in the EF-hand domains of p11 alters its confirmation such that p11 is constantly in the active state, similar to the of S100 proteins upon calcium binding.

The annexin A2 (or p36) subunit is a 36-kDa protein belonging to the annexin family of proteins, a group of calcium-dependent, phospholipid-binding proteins<sup>71-73</sup>. The p36 protein consists of two domains: the carboxy-terminal core region and the amino-

terminal (or head) region. The carboxy-terminal core region is a conserved domain composed of four 70-amino acid segments called annexin repeats. Each annexin repeat contains four  $\alpha$ -helices in an anti-parallel orientation and a fifth  $\alpha$ -helix perpendicular to the four  $\alpha$ -helices, which are all wound in a right-handed superhelix. The carboxy-terminal core region contains calcium-binding sites, and mediates the binding of p36 to anionic phospholipids<sup>76,77</sup>, mRNA<sup>78,79</sup>, F-actin<sup>80</sup>, and heparin<sup>81</sup>. The amino-terminal region is a highly variable domain that mediates ligand and protein interactions as well as post-translational modifications of p36. The amino terminal domain contains serine and tyrosine phosphorylation sites on Ser11<sup>82,83</sup>, Ser25<sup>83</sup>, and Tyr23<sup>84,85</sup>, a reactive cysteine on Cys8<sup>86</sup>, and a nuclear export signal<sup>87</sup>. The p36 protein forms a slightly curved disc with a convex and concave side<sup>71</sup>. The convex side is where calcium and phospholipid binding occurs, and the opposite concave side that faces the cytosol is where the binding of the amino- and carboxy-terminal regions of p11 occurs.

The amino-terminal region of p36 contains an amphipathic  $\alpha$ -helix with a hydrophobic surface that binds p11 to form the AII<sub>t</sub> heterotetramer<sup>71</sup>. Nearly all of the p11 present at the cell surface or within cells is associated with p36<sup>73</sup>. The AII<sub>t</sub> heterotetramer forms intracellularly from a p11 homodimer with each subunit attached to a copy of a p36 subunit (Figure 3). The p11 homodimer interacts with four hydrophobic amino acids (V3, I6, L7, and L10) within the amino-terminal  $\alpha$ -helix of p36. The hydrophobic amino acids on p36 contact seven points with helix HI and two points with the hinge regions on one p11 monomer, and contacts nine points with helix HIV of the other p11 monomer<sup>73</sup>. At the cell surface, p11 is tethered to the cells by the p36 subunits that are bound to the cell surface membrane in a calcium-dependent manner. Similar to

the AIIIt heterotetramer, the convex side of p36 faces the plasma membrane and the concave side faces the cell surface<sup>71</sup>. The convex side of p36 exposes the core region, containing the calcium- and phospholipid binding regions to the cell membrane, and the concave side exposes the amino- and carboxy-terminal regions.

### **1.2.2. Roles of the p11- p36 Protein Interaction**

The p11-p36 interaction is reported to initiate the transport of the AIIIt complex to the cell surface<sup>85,88</sup> and also prevents rapid degradation of newly translated p11<sup>83,89,90</sup>. Before their transport to the cell surface, the p11 and p36 proteins co-localize and bind to each other in the cell cytoplasm, which consequently shields p11 from ubiquitylation and degradation<sup>83,89,90</sup>. Initial observations found that there was a decrease in cellular p11 protein levels associated with the depletion of p36<sup>89</sup>, indicating that p36 maintained the stability of the p11 homodimer. However, the loss of p36 did not change the transcriptional level of p11, indicating that the loss of p11 protein levels is due to a post-translational mechanism. Newly translated p11 has been reported to be rapidly ubiquitylated and degraded by the 26S proteasome<sup>90,91</sup>, but this is prevented by the formation of the AIIIt heterotetramer. The ubiquitylation of p11 was proposed to occur at its carboxy-terminal region on residues 86-95<sup>90</sup>, which are also involved in binding p36. The tumour suppressor deleted in liver cancer 1 (DLC1) competes with p36 for binding to p11 via the carboxy-terminus of p11, the same region that binds p36<sup>92</sup>. Although p11 binding to p36 protects p11 from proteasomal degradation, p11 binding to DLC1 leads to the degradation of p11, again demonstrating that the p36-p11 interaction is critical to the stability of the p11 protein.



P36 is also responsible for the localization of p11 to the cell surface through the formation of the AIIIt heterotetramer. AIIIt attaches to the phospholipids of the plasma membrane, either at the cell surface or intracellularly, by the p36 subunits in a calcium-dependent manner<sup>93,94</sup>. It is not clear how AIIIt is transported to the cell surface as p36 lacks a signal peptide<sup>85</sup>. This indicated that the transport of AIIIt is through an unconventional secretory pathway, which has been supported by demonstrating the involvement of exosomal secretion of AIIIt as p36 has been located on exosomes<sup>95</sup>. Furthermore, p11 binding to p36 increases the latter's affinity for calcium-binding and consequently enhances its association to the plasma membrane<sup>71</sup>. Depletion of p11 by shRNA was shown to prevent the membrane association of p36 indicating the p11-p36 interaction is required for their presence at the cell surface<sup>85</sup>. Post-translation modification of p36 of the AIIIt heterotetramer can also affect AIIIt localization as p36 phosphorylation on Tyr23 is required for the transport of AIIIt to the cell surface<sup>85</sup>.

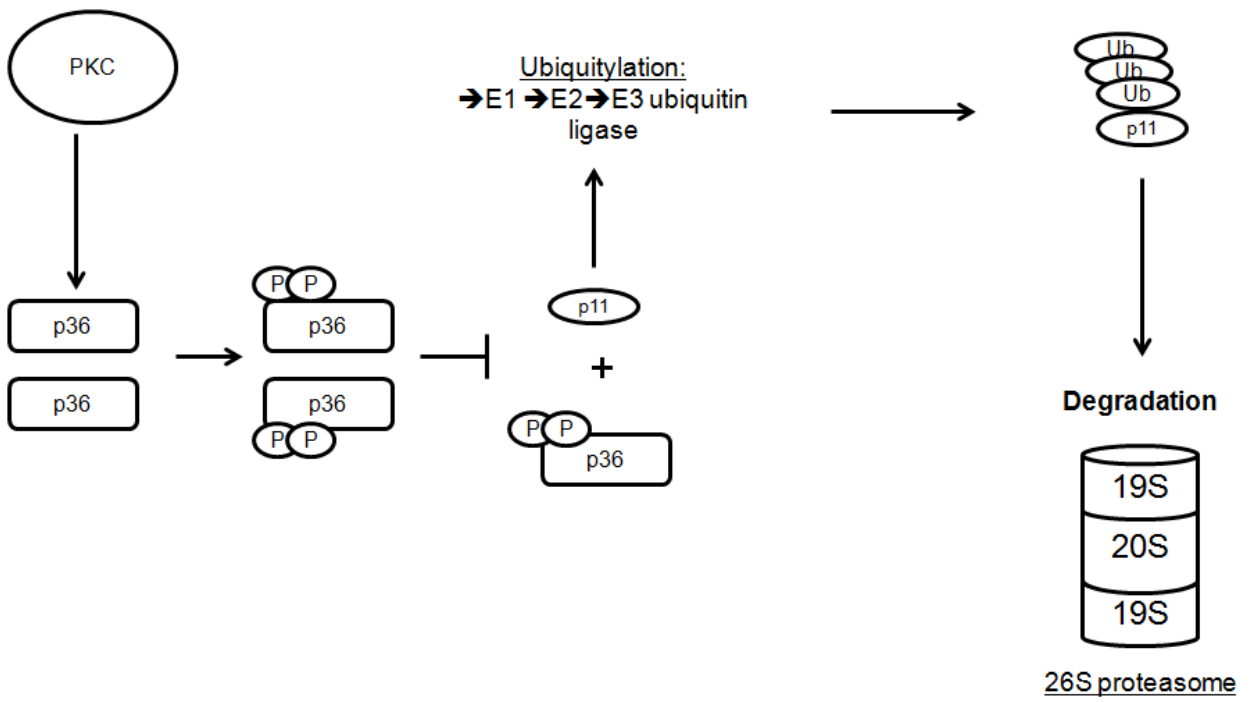
### **1.2.3. P36 Phosphorylation Regulates AIIIt Localization and the p11-p36 Interaction**

The interaction between the p11 and p36 proteins and the localization of the AIIIt heterotetramer has been shown to be regulated through phosphorylation of p36 by Src-kinase and protein kinase C (PKC). P36 phosphorylation regulates its nuclear and cell surface localization<sup>84</sup>, and has been proposed to prevent its interaction with p11<sup>83,96</sup>. Initially, p36 was identified as a phosphorylation substrate of the oncogenic tyrosine kinase v-src during transformation<sup>97,98</sup>. Although this suggested that tyrosine phosphorylation of p36 is up-regulated in cancer, Varmus *et al.*<sup>99</sup> demonstrated that phosphorylated p36 levels were not increased during the transformation of cells with low

expression of v-src. Several growth factors including platelet-derived growth factor (PDGF)<sup>100</sup>, epidermal growth factor (EGF)<sup>101</sup>, and insulin<sup>102,103</sup> induce phosphorylation of p36 on Tyr23 (pY23-p36). The tyrosine phosphorylation of p36 has been reported to mediate p36 localization to plasma membranes as pY23-p36 is associated with endosomes<sup>104</sup>, and is required for transport of AII<sub>t</sub> to the cell surface. This was demonstrated with a Tyr23-mutant of p36 (p36-Y23F) that was incapable of associating with the cell surface<sup>84</sup>. Several studies identified p36 as a target of phosphorylation by conventional, calcium-dependent protein kinase C isoforms ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). PKC-phosphorylation of p36 on Ser25 has been identified *in vivo* and *in vitro*<sup>83,105,106</sup>, but Ser11 phosphorylation has been identified only *in vitro*<sup>107,108</sup>. PKC phosphorylation of p36 on Ser11 and Ser25 has been demonstrated to prevent the formation of the AII<sub>t</sub> complex<sup>83,96</sup> and promote p11 degradation. In human endothelial cells, PKC-dependent phosphorylation of p36 at Ser11 and Ser25 prevented the association with p11, which consequently resulted in the ubiquitylation and proteasomal degradation of p11<sup>83</sup> (Figure 3). Regnoulf *et al.*<sup>96</sup> found that 85% of cellular p36 is monophosphorylated and 15% is diphosphorylated through PKC-serine phosphorylation. They suggested that the phosphorylation on serines 11 and 25 might occur sequentially.

**Figure 3: Regulation of p11 Protein Stability by Protein Kinase C (PKC)-mediated Phosphorylation of p36.**

Protein kinase C (PKC) can phosphorylate p36 on Ser11 and Ser25, which inhibits the protein-protein interaction between p36 and its binding partner, p11. Unpartnered p11 is rapidly ubiquitylated on its carboxy-terminal end and is directed to the 26S proteasome (19S caps and 20S core) for degradation. Abbreviations used: phosphate (P), ubiquitin (Ub).



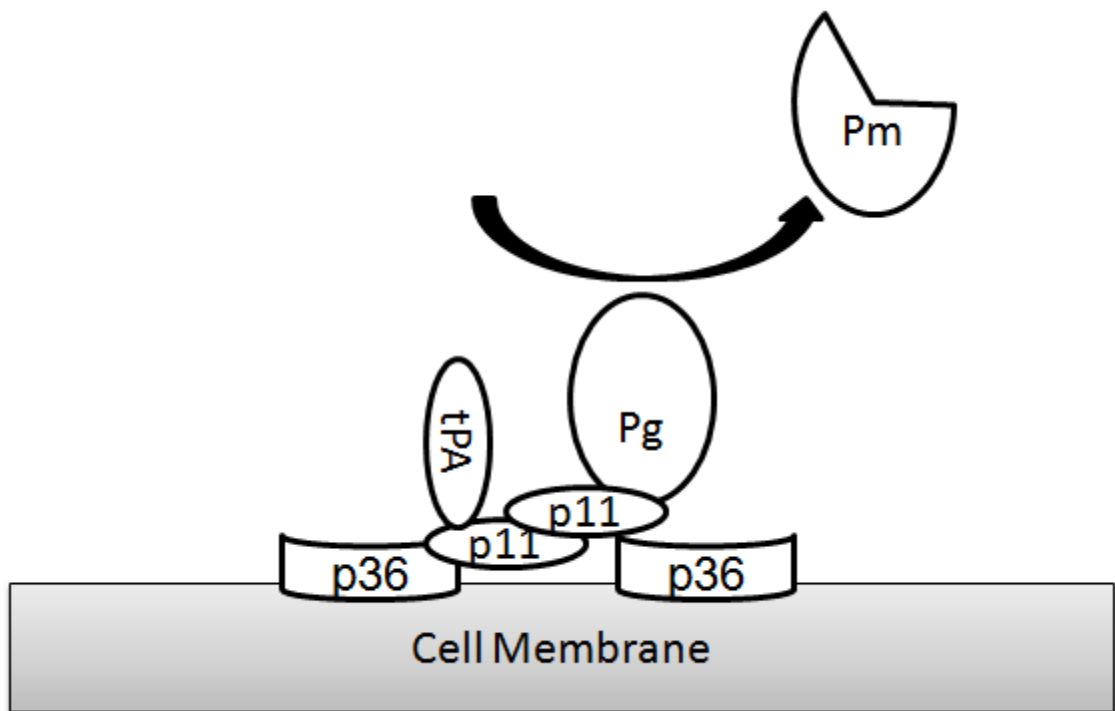
#### 1.2.4. Involvement of p11 in Regulating Plasmin Generation

The p11 subunits of the AIIIt heterotetramer act as a co-receptor for both plasminogen and plasminogen activators: uPA bound to uPAR or tPA (Figure 4)<sup>72,73</sup>. Like most other plasminogen receptors, p11 contains a carboxy-terminal lysine that binds plasminogen and promotes an open form of plasminogen that increases its capability to be activated<sup>73,109</sup>. The carboxy-terminal lysine is also the site that binds plasminogen activators, allowing for the co-localization of plasminogen with tPA or uPA. Recently, the mutation or deletion of several carboxy-terminal lysine residues of p11 was shown to retain its ability to generate plasmin, but at a lower rate than the wildtype p11. This indicated that internal lysine residues are also required for p11 to bind plasminogen and tPA<sup>109</sup>. In addition, the K1 domain of plasminogen and the K2 domain of tPA were shown to be essential for binding to p11. By co-localizing plasminogen and plasminogen activators, the p11 subunit of the AIIIt heterotetramer catalyzes the production of plasmin from plasminogen<sup>71</sup>. As part of the AIIIt heterotetramer, p11 has a high affinity for tPA and plasminogen, in which tPA binds with a  $K_d$  of 0.68  $\mu$ M and plasminogen binds with a  $K_d$  of 0.11  $\mu$ M. The shRNA-depletion of p11 from numerous cell types dramatically reduced plasmin generation. For example, plasmin generation by p11-depleted HT1080 fibrosarcoma cells, was reduced by up to 95% and ECM hydrolysis by these cells was reduced by 70% as compared to HT1080 control cells<sup>110</sup>. Purified annexin proteins, including p36, were shown to have little to no impact on stimulating plasmin generation; however, AIIIt purified from bovine lung was initially reported to dramatically stimulate plasmin generation<sup>111</sup>. It was later found that p11 located at the cell surface was

predominately associated with p36 and this dramatically augmented tPA-dependent plasmin generation<sup>73,112</sup>. The AIIIt heterotetramer stimulated activation of glu-plasminogen by 341-fold, whereas the p36 monomer stimulated activation by only 6-fold<sup>112</sup>, demonstrating that p11, and not p36, was the critical regulator of plasmin generation by AIIIt. Later, tPA-dependent plasmin generation was shown to be stimulated 77-fold by AIIIt, 46-fold by p11 homodimers, and 2-fold by p36 monomers<sup>112</sup>. Furthermore, plasmin generation was similar between AIIIt and AIIIt formed using a truncated form of p36 that consisted of the first 15 amino acids of the p36-amino-terminus containing the p11-binding sites<sup>112</sup>. This strongly indicated that p11 was responsible for the plasmin generation by AIIIt and that the p11-p36 interaction helped stimulate the function of p11. In addition to its role in generating plasmin, p11 also binds newly generated plasmin and protects it from inactivation by  $\alpha_2$ -AP<sup>112</sup>. Plasmin binding to p11 also assists in plasmin-autoproteolysis resulting in production of angiostatins, a group of angiogenesis inhibitors<sup>113</sup>.

**Figure 4: The Regulation of Plasmin Generation by the Annexin A2 Heterotetramer (AIIIt).**

As part of the AIIIt heterotetramer, the p11 homodimer is present at the cell surface by the attachment of membrane-bound p36 to each subunit of p11. The cell surface AIIIt heterotetramer acts as a dual receptor for plasminogen and plasminogen activators, tPA or uPA-bound to uPA receptor (uPAR). By co-localizing plasminogen with tPA or uPA, AIIIt catalyzes the conversion of plasmin from plasminogen.





### 1.2.5. Role of AIIIt in Fibrinolysis and Cellular Migration

The binding of plasminogen activators and plasminogen to p11 on the cell surface promotes their co-localization and catalyzes the production of plasmin<sup>71</sup>. P11-dependent plasmin generation is involved in numerous biological processes including monocyte and macrophage recruitment during the inflammatory response<sup>114,115</sup>, fibrinolysis by endothelial cells<sup>116</sup>, and cancer cell metastasis<sup>110,117,118</sup>.

In endothelial cells, cell surface p11 is a major regulator of plasmin generation that contributes to vascular fibrinolysis. Depletion of p11 by shRNA in the endothelial cell line, telomerase immortalized microvascular endothelial (TIME) cells<sup>119</sup>, dramatically diminished plasminogen binding and plasmin generation by 50% and 60%, respectively, although this did not affect the cell surface levels of p36<sup>116</sup>. Plasminogen binding and plasmin generation were also reduced by depletion of p36 to a similar extent in these cells. Since p36 is necessary for the stabilization and localization of p11, these findings indicate that p11 is a major contributor to the plasmin generation in endothelial cells. *In vivo*, p11-null mice display increased fibrin deposition independent of coagulation as prothrombin time and activated partial thromboplastin time were identical between p11-wildtype (WT) and p11-null mice<sup>116</sup>. A tail-clip experiment was used to characterize the length of time for bleeding to stop between p11-WT and p11-null mice. The p11-null mice showed a 4-fold decrease in the time to stop bleeding compared to the p11-WT group, demonstrating that the loss of p11 resulted in the reduction of plasmin-dependent fibrinolytic activity.

Plasmin generation at the cell surface facilitates the migration of macrophages to the site of inflammation<sup>115</sup> and tumours<sup>114</sup>. Plasmin generated by macrophages is used to

migrate in and out of tissue by hydrolyzing the proteins of the ECM directly and by activating MMPs<sup>120</sup>, which further degrades the ECM. Plasminogen receptors have been shown to contribute to macrophage invasiveness such as histone H2B, Plg-R<sub>KT</sub>,  $\alpha$ -enolase and p11<sup>25,48</sup>. Macrophages isolated from p11-null mice using the thioglycollate-induced peritonitis model showed that the number of macrophages that migrated into the peritoneal cavity was reduced by 53%, and p11-null macrophages displayed limited invasive capability using the Matrigel plug assay *in vivo*<sup>115</sup>. The levels of p36 were decreased in the p11-null mice, and macrophages from p36-null mice also displayed a reduction in p11 levels, plasmin generation and migration. It is unclear whether macrophage migration is affected by the loss of p11, p36 or both; however, p36 was found to be incapable of binding plasminogen or generating plasmin<sup>121</sup>. A role of p11 in mediating recruitment of macrophages to the tumour site was also demonstrated. In a xenograft mouse model where p11-WT and p11-null mice were transplanted with Lewis lung carcinoma (LLC) cells, tumour growth was attenuated after seven days in p11-null mice and tumour growth continued in the p11-WT mice<sup>114</sup>. The tumours formed in the p11-WT mice were 10-fold larger than tumours produced in p11-null mice. Immunohistochemical analysis revealed that macrophages were present throughout the tumour of p11-WT mice, but macrophages were present only at the edge of tumours in p11-null mice, indicating that the loss of p11 impaired the ability of macrophages to infiltrate into the tumour. To confirm that macrophages were responsible for the effect on tumour growth, p11-WT macrophages were peritoneally injected into p11-null mice prior to transplantation of LLC cells to rescue the tumour growth. The injection of p11-WT macrophages into p11-null mice produced tumours comparable to the tumours observed

in p11-WT mice, demonstrating that p11 on the surface of macrophages promotes tumourigenesis.

### **1.2.6. Involvement of AIIIt in Oncogenesis**

Of the plasminogen receptors, p11 of the AIIIt heterotetramer is the most responsive to various stimuli such as physiological signals such as epidermal growth factor (EGF)<sup>122,123</sup> and interferon- $\gamma$ <sup>95,124</sup> and the pathological expression of oncogenes KRAS<sup>117</sup> and PML/RAR $\alpha$ <sup>125</sup>. The role of p11 in promoting invasiveness of tumour cells and metastasis has been reported by numerous studies. In mouse xenograft models, transplantation of p11-depleted and p11-overexpressing HT1080 fibrosarcoma cells show a 3-fold reduction and a 16-fold increase, respectively, in the number of metastatic foci in lungs compared to mice injected with HT1080 cells with wildtype levels of p11<sup>126</sup>. In the colorectal cell line CCL-222, depletion of p11 using shRNA reduced plasminogen binding by 45% and plasmin generation by 65%, and inhibited the migration of these cells through a Matrigel barrier in a plasmin-dependent manner<sup>127</sup>. Gene profiles obtained from circulating tumour cells of breast cancer patients were also shown to have elevated expression of p11 and p36, indicating the correlation between the AIIIt heterotetramer and metastasis<sup>128</sup>.

### 1.3. Acute Promyelocytic Leukemia (APL)

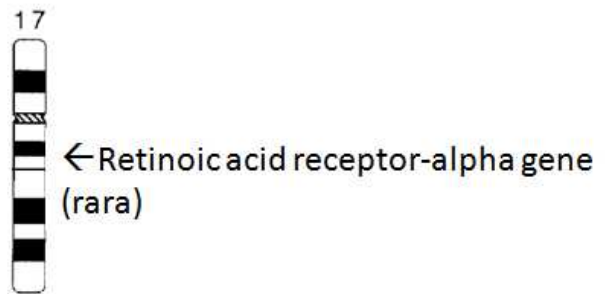
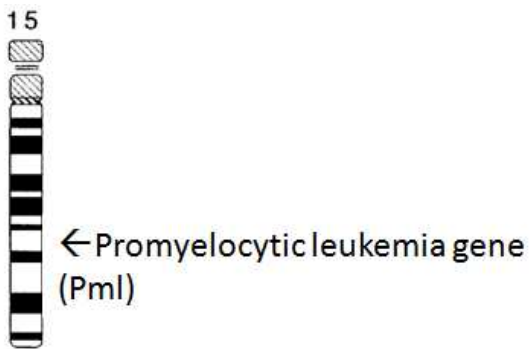
#### 1.3.1. Fusion of the Promyelocytic Leukemia (*Pml*) and Retinoic Acid Receptor- $\alpha$ (*Rar $\alpha$* ) Genes Forms the PML/RAR $\alpha$ Oncoprotein

The chromosomal translocation t(15;17)(q22;q21) fuses the retinoic acid receptor-alpha gene (*Rar $\alpha$* , a nuclear receptor; Discussed further in section 1.4) on chromosome 17 with the promyelocytic leukemia gene (*Pml*) on chromosome 15 (Figure 5; Reviewed in <sup>129</sup>). The *Rar $\alpha$*  gene consists of 9 exons over 7.5 kb and six main domains (A-F) that are evolutionarily conserved<sup>130,131</sup>. The A and B domains form the AF-1 domain, which is involved in ligand-independent transcriptional activation. The C domain contains two zinc-finger motifs that facilitate the binding of RAR $\alpha$  to retinoic acid response elements (RAREs) in the promoters of RAR $\alpha$ -regulated genes. The D domain contains a signal for cellular localization. The E domain is responsible for 1) ligand binding and ligand-dependent activation of RAR $\alpha$  transcription and 2) dimerization of RAR $\alpha$  with retinoid X receptor (RXR), which is necessary for its transcriptional activities. The purpose of the F domain is not understood. The *Pml* gene contains 9 exons over 35 kb that produces multiple transcripts of the gene, with approximately 20 isoforms varying in the carboxy-terminal region of the protein<sup>132</sup>. The PML protein consists of three main domains: the carboxy-terminal domain, the coiled-coil region, and the nuclear localization signal. The carboxy-terminal domain has three zinc-finger domains that mediate the localization of nuclear bodies. The coiled-coil region mediates homodimerization of PML proteins and is also involved in localization of nuclear bodies. The amino-terminal nuclear localization signal mediates the activity of PML by allowing its entry into the nucleus.

The chromosomal translocation of t(15;17)(q22;q21) fuses the *Rara* and *Pml* genes where *Pml* is on the 5'-end and *Rara* is on the 3'-end. The *Rara* portion of *Pml/Rara* fusion gene is truncated at intron two, so that the *Pml/Rara* transcripts produces only domains B-F of the RAR $\alpha$  protein<sup>133</sup>. There are three different breakpoints for the *Pml* gene that occur in intron 6, intron 3 and exon 6. The chromosomal translocation involving the intron 6 breakpoint form of *Pml* (or the long isoform) produces the PML (exon-6)-*Rara* (exon-3) transcript that is found in 70% of the cases of in APL. The chromosomal translocation involving the intron 3 (or the short isoform) produces the *Pml* (exon-3)- *Rara*(exon-3) transcript that accounts for 20% of the cases of APL. The chromosomal translocation involving the exon 6 breakpoint form of *Pml* (or the variable isoform) produces the *Pml*(exon-6)-*Rara*(exon-3) transcript that accounts for 10% of the cases of APL. The chimeric PML/RAR $\alpha$  protein produced is an amino-terminal PML protein with a carboxy-terminal RAR $\alpha$  protein<sup>133</sup>. The RAR $\alpha$  moiety of the PML/RAR $\alpha$  oncoprotein retains features of the RAR $\alpha$  transcription factor including the ability to bind to other RAR $\alpha$ -associated proteins and to interact with RAREs in the promoter regions of RAR $\alpha$ -regulated genes. The presence of PML/RAR $\alpha$  disrupts endogenous RAR $\alpha$  functions and regulation of its target genes, consequently exerting a dominant-negative effect that inhibits the RAR $\alpha$ -mediated differentiation of promyelocytes to granulocytic cell types<sup>134-136</sup>. In addition to impaired promyelocyte differentiation, patients with APL present with an abnormal accumulation of promyelocytes and life-threatening hemorrhagic complications resulting from abnormalities in fibrinolysis<sup>137</sup>.

**Figure 5: Diagram Representing the Chromosomal Translocation That Produces the PML/RAR $\alpha$  oncoprotein.**

Acute promyelocytic leukemia (APL) results from the chromosomal translocation of t(15;17)(q22;q21) fuses the *Rara* gene on chromosome 17 with the *Pml* gene on chromosome 15.



### 1.3.2. Clinical Features of APL

APL is a M3 subtype of acute myeloid leukemia (AML) according to the French-American-British classification<sup>138</sup>, which is associated with a high mortality rate caused by intracranial and pulmonary hemorrhage<sup>136</sup>. APL was initially characterized in 1957<sup>139</sup> as an independent form of leukemia in which normal haematopoiesis was disrupted by the aberrant accumulation of promyelocytes, a precursor of granulocytes. APL occurs in 5-15% of all cases of AML, but is unique from other forms of AML due to a chromosomal translocation that results in the chimeric PML/RAR $\alpha$  oncoprotein. Several U.S.-based institutes reported a relatively low incidence of APL among the total number of cases of AML. The Cancer Surveillance Program of Los Angeles County reported that between 1980-1995, 107 of 2222 cases (4.8%) of AML consisted of APL<sup>140</sup>, and several other U.S.-based institutes reported a 5-13% incidence of APL among AML cases<sup>141</sup>. The incidence of APL is proportional to the age of patients; low incidence of APL in children under the age of 10 years and high incidence in adults over the age of 55 years<sup>141</sup>. Patients with APL present a feature unique from other forms of AML, life-threatening bleeding complications. APL is associated with poor outcome due to early hemorrhagic death<sup>142</sup> occurring in 10-20% of patients<sup>134,135,143</sup>. APL patients present abnormalities in the features of coagulation such as prolonged prothrombin time (the time required for blood to clot by the extrinsic coagulation pathway), thrombin time (the time required for blood to clot in plasma containing excess thrombin and anticoagulant), and partial thromboplastin time (the time required for blood to clot by the intrinsic coagulation pathway)<sup>144</sup>. The hemorrhagic bleeding complications in APL patient is due to abnormalities of two major mechanisms: coagulation and fibrinolysis (Figure 6)<sup>144-146</sup>.



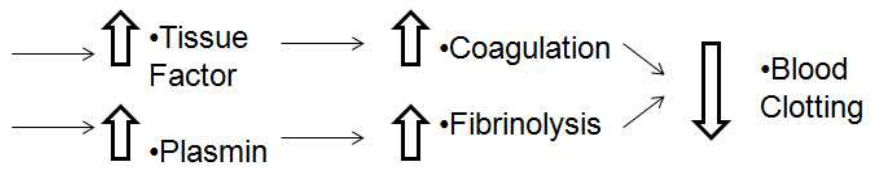
APL patients show evidence of abnormal activation of coagulation in which they present with a low amount of fibrinogen and elevated prothrombin fragments circulating in the blood<sup>137,147,148</sup>. High amounts of fibrin degradation products, including D-dimer<sup>144,149</sup>, are typical in APL patient, thus indicating the increased presence of fibrin caused by the activation of the coagulation cascade. The increase in fibrin degradation products is also indicative of an increase in fibrinolysis due to elevated plasmin activation. Consistent with increased fibrinolysis, the blood of APL patients shows elevated levels of plasmin, plasminogen activators (uPA and tPA), and decreased levels of PAI-1, and  $\alpha_2$ -antiplasmin<sup>150-152</sup>.

**Figure 6: Diagram Representing the Mechanisms Contributing to the Fatal Hemorrhagic Phenotype in APL.**

Aberrant activity of 1) coagulation by excessive tissue factor production and 2) fibrinolysis by excessive production of plasmin in APL promyelocytes are two of the main contributing factors responsible for the fatal hemorrhagic disorder associated with APL.



APL Promyelocyte



### **1.3.3. Deregulation of Coagulation**

One of the key processes contributing to the fatal hemorrhagic phenotype associated with APL is abnormal activation of the coagulation cascade. The abnormal coagulation in APL patients is similar to disseminated intravascular coagulation (DIC), in which rapid activation of intravascular clotting occurs and dramatically consumes coagulation factors circulating in the blood<sup>153</sup>. This effect in APL patients is attributed to the upregulation of the procoagulants, tissue factor (TF)<sup>154-156</sup> and cancer procoagulant (CP)<sup>156</sup>. TF is a transmembrane glycoprotein involved in the activation of the extrinsic coagulation cascade (Described in section 1.2) in both normal and malignant cells<sup>18,157</sup>. APL promyelocytes also produce CP, a cysteine protease and procoagulant expressed predominantly in malignant cells that directly activates FX, but only has a minimal effect of coagulation relative to TF. The aberrant upregulation of TF activity was observed in APL promyelocytes<sup>155,156</sup>, and the induction of PML/RAR $\alpha$  expression in PR9 cells results in an upregulation of TF mRNA levels<sup>158</sup>. Interestingly, TF expression and activity was shown to be regulated by p36 in monocytes<sup>159,160</sup> and in idiopathic pulmonary fibrosis<sup>161</sup>. These studies proposed several mechanisms by which surface p36 regulated TF expression and activity. Since p11 is required for p36 localization to the cell surface<sup>85</sup>, this suggests that the AII $t$  heterotetramer may regulate coagulation as well fibrinolysis in APL.

### **1.3.4. Deregulation of Fibrinolysis**

Another major process contributing to the hemorrhagic complications in APL patients is the excessive production of plasmin<sup>137,146,152</sup>, a significant fibrinolytic factor associated with early deaths in APL patients. This has been indicated by elevated levels

of plasmin, fibrin degradation products, and low levels of plasminogen. APL patients have high levels of free uPA<sup>150</sup> and tPA in their plasma<sup>144,145</sup>. More recently, high expression of both p11 and p36 subunits of the AII<sub>t</sub> heterotetramer have been implicated as the predominant regulator of plasmin generation that is responsible for the hyperfibrinolysis associated with APL cells. Furthermore, APL patients often present intracerebral hemorrhage, which is speculated to be attributed to the high levels of p11 and p36 in the endothelial cells of the cerebral vasculature<sup>162</sup>.

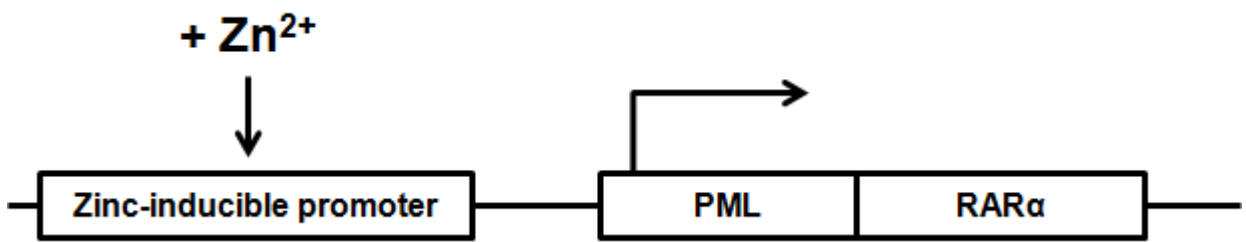
### **1.3.5. Experimental Models of APL**

There are two major *in cellulo* models used to study APL: the patient-derived cell line of APL called NB4, and the U937/PR9 (PR9) cell line that has inducible expression of the PML/RAR $\alpha$  oncoprotein. The NB4 cell line was isolated from the bone marrow of a female patient diagnosed with APL at 20 years of age<sup>163</sup>. The cells were grown on a layer of bone marrow stromal cells for 14 weeks before the NB4 cells, containing the t(15;17) chromosomal translocation, were produced. It was also reported that unlike normal promyelocytes, the NB4 APL cell line could survive in the absence of additional growth factors. Furthermore, cell surface markers were not altered during culture for up to nine months. NB4 cells express surface markers typically observed in APL cells including CD13, CD33, and CD117<sup>163,164</sup>, but they also express some less common surface markers as granulocytic markers (i.e. CD15), T-cell markers (CD 2 and CD4), and monocytic markers like CD9, but not CD14 or CD36<sup>163</sup>. Like APL promyelocytes, NB4 cells are capable of producing excess plasmin and TF. NB4 cells express high levels of uPA and have a high capacity for plasminogen binding compared to other leukemic cell lines<sup>165</sup>, but also express high levels of TF and CP<sup>156</sup>.

The PR9 cells were produced from the U937 cell line derived from a 37-year-old male patient with histiocytic lymphoma<sup>166</sup>. The U937 cells were stably transfected with PML/RAR $\alpha$  cDNA expressed on a zinc-inducible metallothionein promoter (Figure 7)<sup>167</sup>. Under normal/non-induced conditions, PML/RAR $\alpha$  is not detectable in PR9 cells, but PML/RAR $\alpha$  is expressed in the presence of zinc concomitant with the upregulation of plasminogen binding and plasmin generation<sup>125</sup>. Although TF is highly expressed at the protein level in APL and NB4 cells, the induction of PML/RAR $\alpha$  in PR9 cells up-regulated TF at the transcriptional level, but not at the protein level<sup>168</sup>. This indicates that PR9 cells are useful for modelling certain effects mediated by PML/RAR $\alpha$ , but the functional effects induced by PML/RAR $\alpha$ , such as coagulation and fibrinolysis, may not represent the functions consistent with APL cells.

**Figure 7: Model Representing the Zinc-Inducible PML/RAR $\alpha$  Construct of PR9 cells.**

PR9 cells contain a vector that expresses the PML/RAR $\alpha$  cDNA on a zinc-inducible metallothionein promoter; hence, PML/RAR $\alpha$  expression is induced in the presence of zinc.





## 1.4. All-Trans Retinoic Acid Therapy

### 1.4.1. Retinoic Acid Signalling Pathway

The retinoic acid signalling is mediated by ligand binding to retinoic acid receptors that also act as transcription factors and are members of the nuclear receptor family. There are two groups of retinoic acid receptors: retinoid receptors (RAR) and rexinoid receptors (RXR), with each group containing  $\alpha$ ,  $\beta$ , and  $\gamma$  isoforms<sup>169</sup>. The RARs form a heterodimer with RXRs, which binds to retinoic acid response elements (RAREs) located in the promoter regions of numerous genes. The RARE motif is characterized by canonical RARE hexameric repeat [RGKTSA] separated by 1-5-bp direct repeats (DR5)<sup>170,171</sup>. In the absence of ligand, the RAR/RXR heterodimer acts as a transcriptional repressor by forming transcriptional repressive complexes with various nuclear co-repressors<sup>169</sup>. The main co-repressors associated with RARs are histone deacetylases (HDACs), negative co-regulator (N-CoR), and silencing mediator for retinoid and thyroid hormone receptors (SMRT). Derivatives of vitamin A known as retinoids, act as the ligands for the retinoic acid receptors. All-trans-retinoic acid (ATRA) is a ligand for RARs and 9-cis-retinoic acid is a ligand for RARs and RXRs<sup>171</sup>. These retinoids bind to the ligand-binding domain (LBD) of RARs and causes a conformational change of RAR that disrupts its interaction with co-repressors and allows the recruitment of co-activators. These co-activators include histone acetyltransferases (HATs) such as the nuclear co-activators ACTR, CBP/p300, and SCR-1<sup>169,172</sup>.

Retinoic acid signalling is essential to differentiation of myeloid cells and APL promyelocytes. Physiological levels of retinoids in normal promyelocytes induce RAR $\alpha$  to act as a transcriptional activator, which regulates genes involved in myeloid

differentiation<sup>173</sup>. In APL promyelocytes, the RAR $\alpha$  moiety of PML/RAR $\alpha$  fusion protein is present in a constitutively active state in the presence or absence of physiological levels of retinoids<sup>133,136</sup>. Although the RAR $\alpha$  moiety of PML/RAR $\alpha$  fusion protein is constitutively active, the fusion of PML significantly increased the affinity of RAR $\alpha$  for its co-repressors. As a result, physiological levels of retinoids are ineffective in disassociating the co-repressors unless the cells are exposed to pharmacological dosages of ATRA<sup>174</sup>. ATRA induces PML/RAR $\alpha$  and RAR $\alpha$  activation, which is coupled with their proteolytic degradation as a negative feedback mechanism. ATRA was shown to induce PKA phosphorylation of Ser873 on PML/RAR $\alpha$ , which is necessary for the degradation, but not the transactivation of PML/RAR $\alpha$ <sup>175</sup>. ATRA also induces PKA phosphorylation on Ser369 of RAR $\alpha$ , the equivalent residue to Ser873 on PML/RAR $\alpha$ , but phosphorylation of this residue is involved in the transactivation and degradation of RAR $\alpha$ <sup>176</sup>.

Although ATRA exerts transcriptional effects through binding and activating RAR $\alpha$ , it also mediates RAR $\alpha$ -independent effects. ATRA has been shown to stimulate activity of various serine-threonine protein kinases such as members of the mitogen-activated protein kinases (MAPKs)<sup>169</sup>: extracellular signal-regulated kinase (Erk), p38 MAPK, and Jun amino-terminal kinase (Jnk). ATRA was shown to stimulate activation of Erk2 in the HL-60 AML cell line, but not Erk1<sup>177,178</sup>. Similarly, ATRA also stimulated Erk1/2 activity in murine embryonic stem cells, and Erk1/2 activation by ATRA is required for their ATRA-induced differentiation to adipocytes<sup>179</sup>. P38 MAPK was also shown to be activated by ATRA treatment in NB4 cells and MCF-7 breast cancer cells. Interestingly, p38 activation has been implicated in negatively regulating the effect of

ATRA in NB4 cells by opposing ATRA-induced differentiation and cell growth. Alsayed *et al.*<sup>180</sup> examined whether p38 negatively regulated the effects of ATRA in NB4 cells using the p38 inhibitors SB203580 and SB202190, which specifically bind to the ATP-binding region of p38 and block its kinase activity<sup>181</sup>. They found that ATRA treatment combined with SB203580 or SB202190 resulted in the upregulation of CD11b, a marker of myeloid differentiation, and enhanced ATRA-dependent inhibition of cell proliferation. Although p38 inhibition enhanced ATRA-induced differentiation and inhibition of cell proliferation, it had no transcriptional effect on RAR-regulated genes. In contrast to Erk1/2 and p38, ATRA is inhibitory towards Jnk in human bronchial epithelial and non-small cell lung cancer (NSCLC) cells. Studies by Lee *et al.*<sup>182,183</sup> demonstrated that ATRA induced MAPK phosphatase-1 (MKP-1)-dependent dephosphorylation of MAPK kinase 4 (MKK4), an upstream activator of Jnk, and this caused the inhibition of Jnk phosphorylation and activation. More recently, Mathieu *et al.*<sup>184</sup> examined the role of Jnk in ATRA treatment of NB4 cells. Here, ATRA treatment resulted in the activation of Jnk, which was sustained by the induction of reactive oxygen species (ROS)<sup>185,186</sup>. The sustained Jnk activation was shown to contribute to the decreased viability of differentiated cells as NB4 cells treated with a combination of ATRA and the Jnk inhibitor SP600125 improved the viability of the cells.

ATRA is able to directly bind to and activate PKC, but in certain instances, it may also inhibit PKC in a context- and isoform-dependent fashion. PKC has four conserved domains: C1-4<sup>187</sup>. The C1 domain harbours 1-2 Cys-rich motifs that act as the binding site for phorbol esters and diacylglycerol. The C2 domain binds calcium in some PKC isoforms and binds acidic phospholipids. The C3 and C4 domains contain binding sites

for ATP and PKC-substrates, respectively, and together they form the kinase core. Ochoa *et al.*<sup>188</sup> determined that ATRA binds to PKC $\alpha$  through its C2 domain at two locations: the calcium-binding pocket and a lysine-rich region in  $\beta$ -strands three and four. Radomska-Pandya *et al.*<sup>189</sup> reported that ATRA could bind directly to PKC $\alpha$  and decrease its kinase activity *in vitro*. Beradi *et al.*<sup>190</sup> found that ATRA could induce both expression and activity of the PKC $\alpha$  and PKC $\delta$  isoforms in murine LM3 and human SKBR3 mammary cell lines.

#### **1.4.2. Treatment for APL**

Bernard *et al.* first reported the use of anthracyclines in 1973 for the treatment of APL, in which daunorubicin monochemotherapy resulted in a high rate of complete remission<sup>191</sup>. However, APL was still associated with a high mortality rate until 1985 when ATRA treatment was introduced for clinical use<sup>142</sup>. APL is curable through chemotherapy with ATRA<sup>192</sup> and/or arsenic trioxide (ATO)<sup>193,194</sup>. These chemotherapies induce PML/RAR $\alpha$  degradation, restore granulocytic differentiation<sup>195</sup>, and reverse the hemorrhagic disorder present in patients with APL. ATRA therapy has been reported to produce complete remission in patients with APL with a success-rate of over 90%<sup>134,196,197</sup>, which is dramatically higher than the 50-60% complete remission produced by standard treatments anthracyclin and cytarabine<sup>198</sup>. In 1981, Breitman *et al.*<sup>199</sup> first showed that ATRA treatment induced the differentiation of APL promyelocytes to granulocytes *in vitro*. In 1988, Huang *et al.*<sup>200</sup> presented the first evidence that ATRA treatment achieved complete remission in patients with APL wherein all 24 patients in the study showed a reversal of the hemorrhagic disorder. The cause of complete remission was later attributed to be from ATRA-induced differentiation of the APL

promyelocytes to granulocytes<sup>201,202</sup>. Although ATRA treatment results in remission, patients still harbour a small population of APL promyelocytes containing PML/RAR $\alpha$  transcripts<sup>203</sup>, suggesting a probable recurrence of APL promyelocytes. Considering this, it was not surprising that subsequent studies found that APL patients cured by ATRA treatment relapsed at a median of 3.5 months after achieving remission<sup>204,205</sup>. The current treatment regime for APL patients, established by the Gruppo Italiano per le Malattie EMatologiche dell'Adulto (GIMEMA) and the Programa Espanol para el Tratamiento de las HEmopatias Malignas del Adulto (PETHEMA), uses the AIDA protocol. The AIDA protocol consists of anthracycline (idarubicin) chemotherapy in combination with ATRA therapy to induce remission, followed by three cycles of consolidation and maintenance therapy<sup>206,207</sup>. Patients receive 12 mg/m<sup>2</sup> of idarubicin on days 2, 4, 6, and 8, and 45 mg/m<sup>2</sup> of ATRA until remission was achieved. Upon complete remission, patients receive three monthly treatments as consolidation therapy: (1) 4 days of 5 mg/m<sup>2</sup> of idarubicin in the first month, (2) 5 days of 10 mg/m<sup>2</sup> of mitoxantrone in the second month, and (3) 1 day of 12 mg/m<sup>2</sup> of idarubicin in the third month. Maintenance therapy consists of 90 mg/m<sup>2</sup>/day of mercaptopurine, 14 mg/m<sup>2</sup>/week of methotrexate, and 45 mg/m<sup>2</sup>/day of ATRA for 15 days every 3 months.

Similar to ATRA treatment, arsenic trioxide (ATO) also induces the degradation of PML/RAR $\alpha$ . ATO differs from ATRA in that ATO induces apoptosis rather than differentiation, and ATO binds directly to the PML moiety of PML/RAR $\alpha$  on Cys212 and Cys213 of the B2 domain<sup>208</sup>. ATO-binding to the PML moiety then induces oxidation of the cysteine residues leading to the formation of a disulfide bond, which is then followed by sumoylation of Lys160<sup>209,210</sup>. After sumoylation, a reorganization of

PML/RAR $\alpha$  and PML occurs to produce PML-nuclear bodies, which are spherical matrix-associated structures found in most cells and are involved in many regulatory roles including DNA repair<sup>211</sup>, apoptosis<sup>212</sup>, and epigenetic regulation<sup>213</sup>. The ATO-induced formation of the PML-nuclear bodies allows for the polyubiquitylation and degradation of PML/RAR $\alpha$ <sup>208,214</sup>. ATO can cause the degradation of PML/RAR $\alpha$  indirectly by targeting mitochondria and inducing the production of reactive oxygen species (ROS), which promotes the oxidation and degradation of PML/RAR $\alpha$ <sup>136,215</sup>. This was supported by treatment of an APL murine model with  $\alpha$ -tocopheryl succinate ( $\alpha$ -TOS)<sup>215</sup> and paraquat<sup>216</sup> where both compounds produced high levels of ROS. APL was cured by both  $\alpha$ -TOS and paraquat, but paraquat was highly effective in producing ROS and inducing PML/RAR $\alpha$  degradation than  $\alpha$ -TOS treatment. ATO was first demonstrated to be useful as a treatment in humans by Shen *et al.*<sup>194</sup>, in which 9 of 10 patients with APL achieved complete remission. Furthermore, ATO-treated patients were reported to have longer disease-free survival, 87% after 3 years<sup>217</sup> and 80% after 5 years<sup>218</sup>. More recently, Ghavamzadeh *et al.*<sup>219</sup> observed similar trends in long-term complete remission rate in a phase II study where 85% of ATO-treated patients were disease-free after 3 years and 66.7% after 5 years. However, it was noted that a high number of deaths occurred (13.2%) due to a phenomenon known as differentiation syndrome.

Although APL is curable, ATRA- and ATO-treated patients with APL experience a life-threatening complication known as differentiation syndrome, formerly retinoic acid syndrome (RAS). Differentiation syndrome is characterized by the presentation of fever, respiratory distress, pleural and pericardial effusion and pulmonary infiltrates<sup>220</sup>. Tallman

*et al.*<sup>221</sup> reported that 45 of 172 patients with APL (or 26% of patients) experienced differentiation syndrome after a median of 12 days of treatment. Several factors have been found to contribute to differentiation syndrome such as increased pulmonary infiltration of leukemic and maturing myeloid cells<sup>222</sup>, and release of interleukins and vascular factors<sup>223</sup>. Several studies demonstrate that ATRA can enhance plasmin generation by NB4 cells<sup>224,225</sup>, and ATRA-treated patients show increased levels of tPA and lower levels of PAI-1<sup>226</sup>. This suggests that RAS could be associated with the paradoxical upregulation of fibrinolysis.

#### **1.4.3. ATRA Chemotherapy of Non-APL Leukemia and Other Malignancies**

In APL cells, the effect of ATRA in restoring differentiation and reversing the hemorrhagic disorders appears to depend on the effect it has on PML/RAR $\alpha$ . Interestingly, ATRA was shown to induce granulocytic differentiation of an AML cell line, HL-60<sup>227</sup>. The lack of the PML/RAR $\alpha$  fusion protein in this cell line thereby indicated the potential for ATRA treatment in other non-APL AMLs. Venditti *et al.*<sup>228</sup> demonstrated that ATRA treatment in combination with low-dose cytosine arabinoside (LDAC) produces complete remission in 48% of patients with AML. Furthermore, Di Febo *et al.*<sup>229</sup> reported improvement in the survival rate of patients with AML over the age of 60 years when treated with a combination of ATRA and LDAC, but no statistically significant differences were found in complete remission rates between patients treated with LDAC alone or ATRA and LDAC. In contrast to these studies, phase 2 clinical trials have shown no beneficial effects of ATRA in treatment of AML.

The evidence for ATRA treatment in other cancers is also accumulating, and indicates that ATRA can inhibit tumour growth and metastasis in some types of cancers.

In melanoma, ATRA treatment of human M14 melanoma cells inhibited cell adhesion and migration through downregulation of vitronectin receptors<sup>230</sup>. In addition, topical application of ATRA to a xenograft mouse model of melanoma using the B16F10 melanoma cells reduced the growth of melanoma by stimulating effector CD8+ T-cell responses<sup>231</sup>. Centritto *et al.*,<sup>232</sup> profiled the sensitivity of 42 breast cancer cell lines to ATRA treatment and found that luminal and ER+ type breast cancer cell lines were most sensitive to ATRA-induced growth inhibition and basal breast cancer cells were more resistant. They showed that the sensitivity of HCC-1599c breast cancer cells to ATRA was also supported in subcutaneous xenotransplanted SCID mice, where ATRA treatment reduced tumour growth in a dose-dependent manner. Additionally, cultures of patient tumour slices treated with ATRA show that ATRA reduced the expression of Ki67, a marker of cell proliferation, in luminal breast tumours (characterized by having >70% ER<sup>+</sup> cells), but ATRA did not affect Ki67 levels in triple negative (PR<sup>-</sup>, ER<sup>-</sup>, HER2<sup>-</sup>) basal breast tumours<sup>232</sup>. ATRA treatment of lung cancer patients does not always produce beneficial effects, which has been attributed to dysregulation of Erk1/2 signalling. Because ATRA can stimulate Erk1/2 signalling, ATRA treatment promotes the proliferation, survival and migration of lung cancer cells<sup>233</sup>. However, Erk1/2 inhibition in combination with ATRA restores the ability of ATRA to inhibit the proliferation, survival and migration of lung cancer cells.



## **1.5. Proteasomal Degradation Pathways**

### **1.5.1. Proteasome Structure and Function**

Eukaryotic cells use the proteasome, a large protein complex located in the cytoplasm as well as the nucleus, for the proteolytic degradation of damaged or unwanted proteins. Approximately 80-90% of cellular proteolysis is due to degradation by proteasomal complexes. These complexes consist of the barrel-like 20S core complex (700 kDa) present by itself or with the 19S cap (900 kDa) on one or both ends of the 20S core<sup>234,235</sup>. The subunits of the 20S proteasome core complex consists of four sets of stack rings where the two inner set of rings are responsible for the proteolytic activities of the proteasome. The two sets of inner rings are each composed of seven  $\beta$ -subunits and the outer rings at the ends of the 20S proteasome are composed of seven  $\alpha$ -subunits. The inner rings of  $\beta$ -subunits, specifically  $\beta 1$ ,  $\beta 2$  and  $\beta 5$ , are responsible for the proteolytic function of the proteasome through trypsin-like, chymotrypsin-like, and peptidyl-glutamyl activities. The  $\alpha$ -rings are capable of binding substrates, which induces a conformational change in the structure of the  $\alpha$ -subunits, allowing for the translocation of substrates into the inner cavity of the 20S proteasome for degradation. The  $\alpha$ -rings are also the sites where accessory complexes, such as the 19S cap, bind to the 20S proteasome to mediate the specificity of proteins marked for degradation by post-translational modifications, mainly ubiquitin or ubiquitin-like proteins.

### **1.5.2. Ubiquitylation**

Some mechanisms of protein degradation by proteasome complexes require a post-translational modification of ubiquitin, a small protein that typically targets proteins for proteasomal degradation<sup>236</sup>. Ubiquitin is a small ubiquitously expressed 8.5 kDa

protein that can be attached to substrate proteins in an ATP-dependent manner. Ubiquitin is attached to substrate proteins via their lysine residues through a series of enzymatic reactions referred to as ubiquitinylation<sup>237</sup>. Ubiquitinylation of proteins occurs through three types of enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases (Figure 8). Ubiquitylation begins by E1 ubiquitin-activating enzymes binding ATP, which allows ubiquitin to bind to the carboxy-terminus of ATP. This leads to the transfer of ubiquitin to the E1 ubiquitin-activating enzyme in which ubiquitin is covalently attached through the formation of a thioester bond on a cysteine residue (Cys632). The E1-ubiquitin complex can transfer ubiquitin to E2 ubiquitin-conjugating enzymes on a cysteine residue, a process called transthioesterification. Ubiquitin-conjugated E2 enzymes are now able to transfer ubiquitin to the lysine residues of substrate proteins directly or by transferring ubiquitin to E3 ubiquitin ligases. Ubiquitin is covalently attached by its carboxy-terminal diglycine residues to the amino group of a lysine residue within the substrate protein.

Ubiquitylation of protein can occur as the addition of a single ubiquitin subunit at one (monoubiquitylation) or multiple sites (multiubiquitylation), or the ubiquitin can be added on to a single ubiquitin attached to a substrate to produce a chain of multiple ubiquitin subunits (poly-ubiquitylation)<sup>236</sup>. Polyubiquitylation of proteins is well-known to be involved as a key signal for the destruction of the protein by proteasomal degradation; however, mono/multi-ubiquitylation can also be involved in the alteration of protein function or localization.

Monoubiquitylation of substrate proteins typically mediates non-degradation functions, such as modulation of protein activity, interaction with other proteins and

trafficking, but the attachment of a single ubiquitin to one or more sites on a protein can initiate protein degradation as well<sup>236</sup>. Monoubiquitylation was shown to mediate the protein trafficking as monoubiquitylated-p53 promoted its nuclear export<sup>238</sup> and monoubiquitylation of phosphatase and tensin homolog on chromosome ten (PTEN) mediates its localization to the nucleus<sup>239</sup>. Histone ubiquitylation is an important regulator of transcriptional activity and protein interactions. Histone H2B monoubiquitylation on Lys120 promotes gene activation and transcription elongation, whereas Histone H2A monoubiquitylation on Lys119 inhibits its transcription activity by preventing the **f**acilitates **c**hromatin **t**ranscription (FACT) complex from displacing histones<sup>240</sup>. In a proteomics study<sup>241</sup>, cells were engineered to express a mutant ubiquitin with all the lysines changed to arginine to prevent the formation of polyubiquitin chains. The findings of this study indicated that approximately 50% of proteins in human cells are degraded following mono- or multi-ubiquitylation. Furthermore, the proteins that are degraded tend to be small (approximately 150 residues) and have disordered structures. Multiubiquitylation was reported to mediate the endocytosis and lysosomal degradation of the receptors of EGF and PDGF<sup>242</sup>.

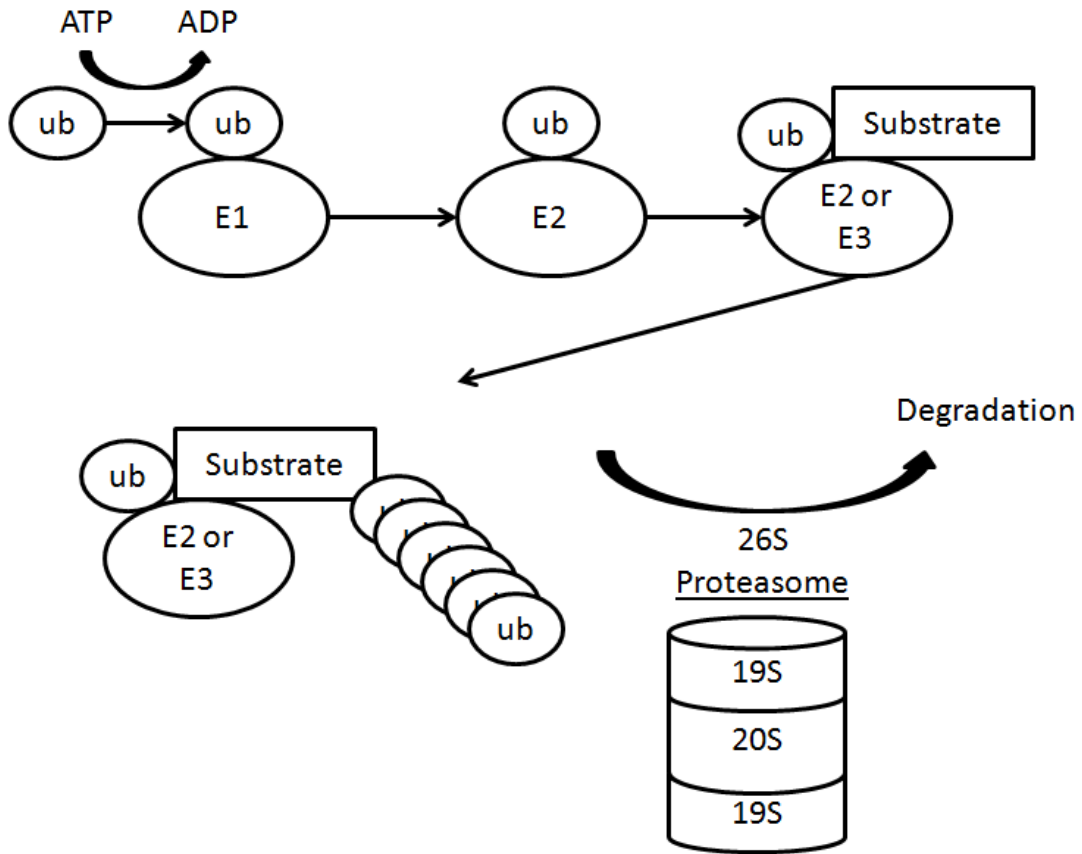
After the initial attachment of ubiquitin to the substrate protein, additional ubiquitin subunits can be covalently attached to the substrate-bound ubiquitin at lysine residues (Lys6, 11, 27, 29, 33, 48, and 63) or at the amino-terminal methionine (Met1)<sup>236</sup>. The most abundant (or typical) linkages of ubiquitins to the lysine residues are through Lys48, which contributes to half of all ubiquitin linkages, and Lys63. Other linkages are less abundant (atypical), but their abundance can be greatly elevated under specific conditions such as proteasomal inhibition or due to various stresses. Polyubiquitylation

through Lys48 linkages typically signals a substrate for proteasomal degradation and targets over 5000 cellular proteins<sup>236,237</sup>. Protein degradation by Lys48 polyubiquitin linkages affects most biological processes of the cell, including cell division, transcription, and signalling pathways<sup>243-245</sup>. Polyubiquitylation through Lys63 linkages plays a role in targeting proteins associated with cellular materials, such as pathogens or damaged organelles, for degradation via autophagy<sup>246</sup>, but can also mediate non-degradation activities including protein transport<sup>247,248</sup>, and protein kinase activity<sup>249</sup>. Lys11 polyubiquitin chains are the most abundant linkage among the atypical polyubiquitin chains depending on physiological conditions<sup>250,251</sup>. Polyubiquitin chains linked through Lys11 tend to be found as mixed or branched chains that also contain Lys48 and Lys63 linkages, and target substrate proteins for proteasomal degradation<sup>252,253</sup>. Lys29 polyubiquitin chains are abundant among the atypical polyubiquitin linkages in non-stressed cells and have been found on the amino-terminus of substrates rather than lysine residues<sup>254,255</sup>. In yeast, Lys48 linkages have been found on Lys29 polyubiquitin chains and mediate proteasomal degradation of substrate proteins<sup>256</sup>.

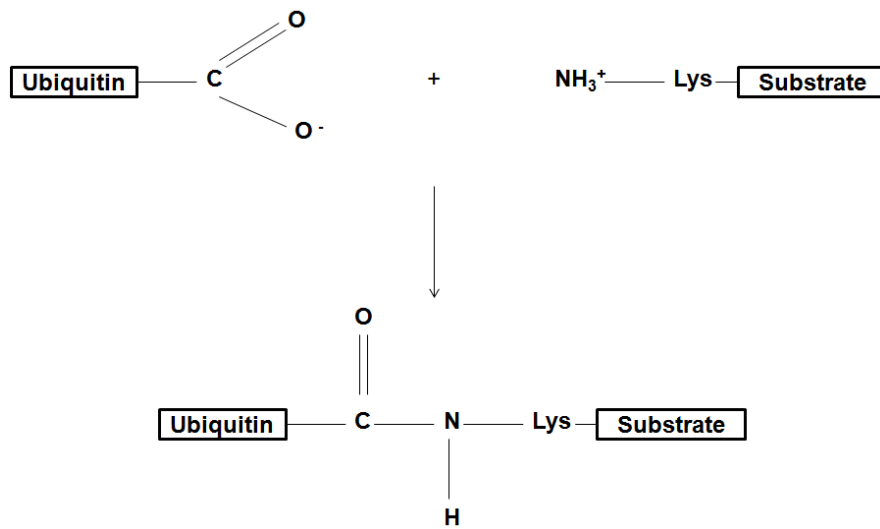
**Figure 8: The Ubiquitylation-Proteasome Pathway.**

(A) Ubiquitylation directs proteins for degradation by the 26S proteasome by the covalent attachment of the small protein, ubiquitin, to the substrate. This post-translational modification of ubiquitin is mediated by the ubiquitin-activating enzyme (E1), ubiquitin-transferring enzymes (E2), and ubiquitin ligases (E3). The E1 activates ubiquitin in an ATP-dependent manner and ubiquitin is attached to the E1 enzyme by a thioester bond. Ubiquitin is then attached to an E2 enzyme by a thioester bond. E2 enzymes can either transfer ubiquitin onto lysine residues of an E3 enzyme or transfer ubiquitin directly to the lysine residues of substrate proteins. After the initial attachment, additional ubiquitin subunits can be covalently attached to the substrate-bound ubiquitin at lysine residues (Lys6, 11, 27, 29, 33, 48, and 63) or at the amino-terminal methionine (Met1). In most cases, the formation of polyubiquitin chains direct the substrate protein to the 19S cap of the proteasome for degradation. (B) The carboxy-terminal diglycine residues of ubiquitin form an isopeptid bond at the  $\epsilon$ -amino group of a lysine residue of substrate proteins.

A.



B.



### 1.5.3. The 26S Proteasome (Ubiquitin-dependent)

Ubiquitin-dependent proteasomal degradation is mediated by the 26S proteasome complex that consists of the 20S core and the 19S cap, which is present on one of the two ends of the 20S core complex (Reviewed in <sup>234</sup>). The 19S cap is responsible for the ability of the 26S proteasome to recognize and degrade polyubiquitylated proteins. The ubiquitin-dependent proteasomal pathway (UPP) regulates the degradation of damaged proteins or those that exist for a short period. The subunits of the 19S cap complex are involved in the recognition of polyubiquitylated proteins. Once the ubiquitylated protein interacts with the 19S cap complex, isopeptidases remove ubiquitin and unfold the substrate protein prior to passing the unfolded protein into the inner chamber of the 20S core proteasome where the protein is proteolyzed into oligopeptides.

The 19S cap is a highly conserved complex unique to eukaryotes composed of a nine-subunit base, a nine-subunit lid, and the Rpn10 subunit<sup>235</sup>. The base of the 19S cap consists of six AAA ATPases (Rpt1-6) and three non-ATPase (Rpn1, Rpn2, and Rpn13). Each Rpt subunit contains four domains: the amino-terminal  $\alpha$ -helix, the oligonucleotide/oligosaccharide-binding fold, the large ATPase domain, and the carboxy-terminal helical domain. The Rpt subunits associate as dimers (Rpt1-Rpt2, Rpt6-Rpt3, and Rpt4-Rpt5) and form a trimer of dimers. The Rpt subunits play a role in unfolding ubiquitylated proteins and opening the channel to transport proteins for degradation inside the 20S core. The Rpn1 and Rpn2 subunits are bound to Rpt1/2 and Rpt6/3 respectively. The Rpn1 and Rpn2 proteins consist of 11 proteasome/cyclosome (PC) repeats flanked by an amino-terminal domain of stack helices and a carboxy-terminal domain of globular B-sandwiches. Rpn13 binds to Rpt2 and has two distinct domains: the

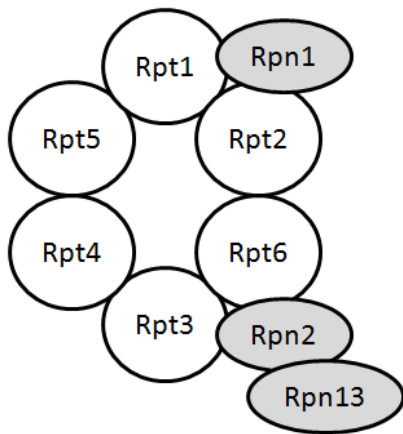
conserved amino-terminal pleckstrin-like receptor for ubiquitin (PRU) domain and the carboxy-terminal domain responsible for binding the de-ubiquitylation enzyme Uch37. Rpn1, Rpn2 and Rpn13 are able to recognize and bind ubiquitylated substrates. The lid of the 19S cap is composed of nine subunits (Rpn3, 5-9, 11, 12, 15), which are proposed to be mainly involved in the de-ubiquitylation of proteins. The Rpn3, 5, 6, 7, 9 and 12 subunits are structurally similar to the COP9 signalsome and eIF3 and are referred to as the PCI subunits. The PCI subunits form horseshoe-like formation, Rpn15 binds to both Rpn3 and Rpn7 subunit, and the Rpn8-Rpn11 heterodimer bind to Rpn9 and Rpn5, respectively (Figure 9). The final component of the 19S cap is the Rpn10 subunit, which is responsible for stabilizing the interaction between the lid and the base, and acts as one of the ubiquitin receptors found in the 19S cap.



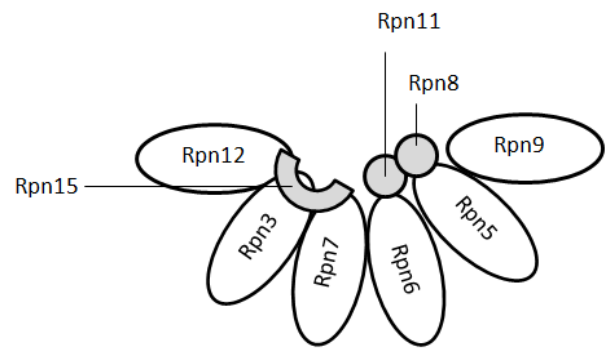
**Figure 9: Structure of the 19S Cap of the 26S Proteasome.**

The 19S cap is composed of the base, the lid, and the Rpn10 subunit. (A) The base consists of six AAA ATPases (Rpt1-6) and three non-ATPase (Rpn1, 2, 13), where the Rpt subunits associate as a trimer of dimers (Rpt1-Rpt2, Rpt6-Rpt3, Rpt4-Rpt5) and the Rpn1 and Rpn2 subunits are bound to Rpt1/2 and Rpt6/3 respectively. Rpn13 is attached to Rpn2. (B) The lid is composed of nine subunits (Rpn3, 5-9, 11, 12, 15), which are proposed to be mainly involved in the de-ubiquitylation of proteins. The Rpn 3, 5, 6, 7, 9 and 12 subunits (the PCI subunit) form horseshoe-like formation. Rpn15 binds to both Rpn3 and Rpn7 subunit, and the Rpn8-Rpn11 heterodimer bind to Rpn9 and Rpn5, respectively

A



B



#### 1.5.4. The 20S Proteasome (Ubiquitin-independent)

The 20S proteasome complex is the core of all proteasome complexes found in the cytoplasm and nucleus and represents 1% of the total protein in cells<sup>257</sup>. In a variety of cell types, 64% of the 20S proteasome complexes within cells are found without any additional structures, 26% are associated with the 19S regulatory cap or other regulatory complexes such as the 11S cap, PA29g, and PA200<sup>257,258</sup>. Proteasomal degradation by the 20S proteasome can occur independent of ubiquitylation, in which proteins can interact directly with the 20S proteasome or are delivered to it by a chaperone, and are degraded directly through its proteolytic activities<sup>257</sup>. Substrates of the 20S proteasome are typically proteins that are partially or completely unfolded caused by factors including aging, oxidation and mutations, or proteins that contain unstructured regions known as intrinsically disordered domains (IDDs)<sup>259</sup> with exposed hydrophobic regions<sup>260</sup>. Hydrophobic residues of these unstructured regions can bind to the  $\alpha$ -subunit of the 20S proteasome and induce the opening of the  $\alpha$ -rings to allow the translocation of the protein into the interior of the proteasome for degradation<sup>261</sup>. Tsvetkov *et al.*<sup>262</sup> demonstrated *in vitro* that the 20S proteasome digests unstructured proteins, but structured proteins are resistant to proteasomal degradation. Furthermore, they show that protein-protein interaction with unstructured protein can block their degradation. For example, NAD(P)H:quinone oxidoreductase 1 (NQO1) was found to compete with the 20S proteasome for binding of numerous intrinsically unstructured proteins such as C/EBP $\alpha$ , p53 and p73 preventing their degradation<sup>263,264</sup>.

## 1.6. Conceptual Framework

The objective of this study was to characterize the mechanisms by which p11 is regulated in APL and by ATRA therapy. Freshly translated p11 is only stable after forming a complex with p36; hence, this complicates the understanding of the mechanisms regulating p11 by the PML/RAR $\alpha$  oncoprotein in APL and treatment by ATRA. In APL, the expression of the PML/RAR $\alpha$  oncoprotein up-regulates both p11 and p36 protein levels, but it is not certain if p11 is highly expressed due to the overexpression of p36 or p11 protein. Likewise, ATRA produces a loss of p11 and p36 protein levels, but it is not certain if p11 expression is lost due to the loss of p36 or p11 protein. Therefore, treatments that modulate p36 protein levels will affect p11 protein levels. The prevailing hypothesis proposes that in the absence of p36 or with disruption of the p11-p36 interaction, the p11 protein is unstable due to rapid polyubiquitylation on carboxy-terminal lysines, and is degraded by the 26S proteasome. However, our preliminary findings failed to detect ubiquitylated p11 in ATRA-treated NB4 cells. Hence, we hypothesize that the ATRA-dependent degradation of p11 does not involve ubiquitin-mediated degradation by the 26S proteasome.

A secondary objective of this study aimed to re-evaluate the role of p11 in the hyperfibrinolytic disorder of APL. ATRA treatment of APL promyelocytes induces the loss of p11 protein levels; however, the effect of ATRA on plasmin generation has been contested by several studies since the initial report by Menell *et al.*<sup>146</sup>, which indicated that ATRA or p36-blocking antibodies attenuated the excessive production of plasmin in APL promyelocytes. Hence, depletion of p11 using ATRA or ATO treatments, or shRNA was used to assess the contribution of p11 to the excessive plasmin generation associated

with APL. Here, we hypothesize that the loss of p11 will produce a reduction of plasmin generation by APL promyelocytes.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1. Materials**

#### **2.1.1. Reagents**

All-trans retinoic acid (ATRA), arsenic trioxide (ATO), zinc sulfate ( $ZnSO_4$ ), MDL28170 (calpain inhibitor III), and ammonium chloride ( $NH_4Cl$ ) were purchased from Sigma-Aldrich (Oakville, ON, Canada). 4-nitroblue tetrazolium was purchased from Fisher Scientific (Ottawa, ON, Canada). GF109203X and lactacystin were purchased from Enzo Life Sciences (East Farmingdale, NY, USA). Gö6983 and phorbol 12-myristate 13-acetate (PMA) were purchased from Tocris (Minneapolis, MN, USA). PYR-41 was purchased from BioVision (Milpitas, CA USA). Purified 20S proteasome was purchased from Boston Biochemical (Boston, MA, USA) or Enzo Life Sciences. Calpain inhibitor IV was purchased from MilliporeSigma (Etobicoke, ON, Canada). Table 2 summarizes the reagents used to treat cells and their purpose in these studies.

**Table 2: List of reagents used and their purpose.**

<b>Reagents</b>	<b>Purpose</b>
ATRA	Stimulates degradation of PML/RAR $\alpha$ in APL cells. Acts as a ligand for RAR $\alpha$ .
ATO	Stimulates degradation of PML/RAR $\alpha$ in APL cells
GF109203X	Inhibitor of PKC- $\alpha$ , - $\beta$ , and - $\delta$ isoforms
Gö6983	Inhibitor of PKC-- $\alpha$ , - $\beta$ , - $\delta$ , - $\gamma$ , and - $\zeta$ isoforms
Lactacystin	Inhibitor of the 20S core of the proteasome
PYR-41	Inhibitor of the E1 ubiquitin-activating enzyme
Calpain inhibitor IV	Irreversible inhibitor of calpain II, cathepsin L, proteasome
MDL28170	Inhibitor of calpain I & II and cathepsin B
NH <sub>4</sub> Cl	Inhibitor of lysosomal degradation
ZnSO <sub>4</sub>	Used to activate the PR9 vector that expresses the PML/RAR $\alpha$ oncoprotein on a zinc-inducible promoter

### **2.1.2. Anti-Sera**

The primary antibodies used for western blotting and their working dilutions are listed in Table 3. The secondary antibodies used were the IRdye-800 goat anti-mouse antibody (1:10,000 dilution; LI-COR Biosciences, Lincoln, NE, USA) and IRdye-680 goat anti-rabbit antibody (1:5,000 dilution; Fisher Scientific).



**Table 3: A list of the primary antibodies used in western blot analysis.**

Antibody	Working Dilution	Company
Anti-human p11	1: 1,000	BD Biosciences (San Jose, CA, USA)
Anti-human p36	1: 1,000	BD Biosciences
Anti-human RAR $\alpha$	1: 500	Santa Cruz (Mississauga, ON, Canada), C-20
Anti-human ubiquitin	1: 1,000	Cell Signaling (Danvers, MA, USA)
Anti-human cyclin D1	1:1,000	Cell Signaling
Anti-phospho(serine)-PKC substrates	1:1,000	Cell Signaling
Anti- $\alpha$ -enolase	1:1,000	Abcam (Toronto, ON, Canada), ab54979
Anti-cytokeratin-8	1:25,000	Abcam, ab53280
Anti- HA	1:1,000	Cell Signaling
Anti-histone H2B	10,000	Abcam, ab52599
Anti-HMGB1	1:1,000	Sigma, 649537
Anti-Plg-R <sub>KT</sub>	1:5,000-8,000	A kind gift from Dr. Lindsey Miles
Anti-S100A4	1:200	Abcam
Anti-human tissue factor	1:1,000	Cell Signaling
Anti-human $\beta$ -actin	1: 10,000	Sigma

## **2.2. Mice**

Mice were maintained on a 12-hour light cycle in the Carleton Animal Care Facility at Dalhousie University where they were fed standard rodent chow and watered *ad libitum*. The p36-deficient mice (p36<sup>-/-</sup>) and their wildtype counterparts (p36<sup>+/+</sup>) are on a 129SV x C57BL/6 background, and were a generous gift from Dr. Katherine Hajjar. Experimental mice were typically 6-8 weeks old of both genders. Mice were sacrificed by CO<sub>2</sub> asphyxiation to isolate peritoneal macrophages for protein analysis. All animal protocols were approved by the University Committee on Laboratory Animals (UCLA).

## **2.3. Isolation of Thioglycollate-elicited Primary Peritoneal Macrophages**

Mice received an intraperitoneal injection of 3 mL sterile 4% Brewer's thioglycollate broth medium (Sigma-Aldrich). Five days post-injection, mice were sacrificed and peritoneal cells were isolated by lavage using 5 mL of complete RPMI-1640 media (Invitrogen/Thermo-Fisher, Ottawa, ON, Canada) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (P/S) and kept on ice. Lavage fluids were centrifuged at 1,250 rpm for 5 min at room temperature and supernatants were discarded. Cells were resuspended in complete RPMI-1640 media, and seeded to 10-cm<sup>3</sup> culture dishes. Macrophages were further purified by allowing cells from lavage fluids to adhere to the culture dish for ~16 h under standard culture conditions (at 37°C in a humidified atmosphere and 5% CO<sub>2</sub>), followed by media replacement to remove the non-adherent and non-macrophage cells.

## **2.4. Cell Culture**

### **2.4.1. Cell Lines and Culture Methods**

NB4-MR2 and U937/PR9 cells, which express PML/RAR $\alpha$  under the control of the zinc-inducible promoter cells, (kindly provided by Dr. Wilson Miller Jr., McGill University, Montreal, QC, Canada), NB4 cells (DSMZ, Braunschweig, Germany), U937 cells (ATCC, Manassas, VA, USA) were maintained in RPMI-1640 medium (Invitrogen/Thermo-Fisher) supplemented with 10% FBS and 1% penicillin/streptomycin under standard culture conditions (at 37°C in a humidified atmosphere and 5% CO<sub>2</sub>). MCF-7, MBA-MD-231, SUM159PT (kindly provided by Dr. Paola Marcato, Department of Pathology, Dalhousie University, Halifax, NS), phoenix and HEK293T cells (ATCC) were maintained in DMEM (Invitrogen/Thermo-Fisher) supplemented 0% FBS and 1% penicillin/streptomycin under standard culture conditions (at 37°C in a humidified atmosphere and 5% CO<sub>2</sub>). Non-adherent cells were maintained at a cell density of  $< 1 \times 10^6$  cells/mL and adherent cells were seeded at a cell density of  $0.35 \times 10^6$  cells in 6-well dishes.

### **2.4.2. ATRA treatment**

Non-adherent cell lines (NB4, U937, U937/PR9 cells) in their exponential growth phase were used to start the suspension culture at a density of  $0.3 \times 10^6$  cells/mL before exposure to ATRA. Adherent cell lines (MCF-7, MDA-MD-231, SUM159PT cells) in their exponential growth phase were used to start cultures at a density of  $0.25 \times 10^6$  cells/well of 6-well plates before exposure to ATRA. Stock solutions of 50 mM ATRA (Sigma-Aldrich) were diluted in DMSO (Sigma-Aldrich) to 5 mM and added to the cells to achieve a final concentration of 1  $\mu$ M. Cells were grown in medium

containing 1  $\mu$ M ATRA added daily over the course of the indicated times. Control treatments had an equal volume of DMSO added. Cell densities of suspended cells was maintained at  $< 1 \times 10^6$  cells/mL throughout the experiment.

### **2.4.3. PML/RAR $\alpha$ induction of U937/PR9 cells**

U937/PR9 cells (seeded at  $0.3-0.5 \times 10^6$  cells/mL) were treated a day after seeding cells using 100  $\mu$ M ZnSO<sub>4</sub> (Sigma-Aldrich) added daily over the course of the indicated times. Cell density was maintained at  $< 1 \times 10^6$  cells/mL throughout the experiment.

## **2.5. Production of Plasmid DNA**

### **2.5.1. Plasmids**

The cDNA for full-length *p11* was amplified by PCR and ligated into the pcDNA3.1/neomycin (pcDNA-p11) vector (Invitrogen) that constitutively expresses high levels of p11 under the control of the SV40 promoter. The pRK5 vectors expressing wildtype or mutant HA-tagged ubiquitin (K48R, K48only, and K0) were purchased from Addgene. The pSUPER-retro-p11 shRNA1 vector was constructed by cloning the dsDNA oligo 5'-GAT CCC CGT GGG CTT CCA GAG CTT CTT TCA AGA GAA GAA GCT CTG GAA GCC CAC TTT TTA-3' and 5'-AGC TTA AAA AGT GGG CTT CCA GAG CTT CTT CTC TTG AAA GAA GCT CTG GAA GCC CAC GGG-3' into pSUPER-retro-puro (OligoEngine). The pSUPER-retro-p36 shRNA1 vector was constructed by cloning the dsDNA oligo 5'-GAT CCC CCC TGG TTC AGT GCA TTC AGT TCA AGA GAC TGA ATG CAC TGA ACC AGG TTT TTA-3' and 5'-AGC TTA AAA ACC TGG TTC AGT GCA TTC AGT CTC TTG AAC TGA ATG CAC TGA

ACC AGG GGG-3' into pSUPER.retro-puro (OligoEngine), and the pSUPER-retro scramble was constructed by cloning the dsDNA oligo 5'-GAT CCC CGT GGG AGT TCA GAG CTT CTT TCA AGA GAA GAA GCT CTG AAC TCC CAC TTT TTA-3' and 5'-AGC TTA AAA AGT GGG AGT TCA GAG CTT CTT CTC TTG AAA GAA GCT CTG AAC TCC CAC GGG-3' into pSUPER.retro-puro (OligoEngine).

### **2.5.2. Transformation of competent *E. coli* cells**

Competent DH5a *E. coli* (Invitrogen) were thawed on ice. The plasmid to be amplified (200 ng) was added to 50  $\mu$ L of *E. coli* cells, which were kept on ice for 30 minutes. The *E. coli* cells were heat shocked for 90 seconds at 42°C, immediately placed on ice, transferred to 1 mL of Luria-Bertani (LB) medium [1% (w/v) peptone, 0.5% yeast extract and 1% NaCl] containing 50  $\mu$ g/mL ampicillin and incubated for 1 h at 37°C with shaking (225 rpm). Subsequently, transformed bacteria were selected on LB-agar plates (LB medium with 1.5% Bacto Agar) supplemented with 100  $\mu$ g/mL ampicillin. The bacteria were incubated for 12-16 h at 37°C to allow colony formation.

### **2.5.3. Plasmid DNA Purification**

*E. coli* cultures were grown overnight at 37°C with shaking (225 rpm) in 500 mL LB medium supplemented with 100  $\mu$ g/mL ampicillin. The next day, the bacterial cultures were centrifuged for 10 min at 5,000  $\times$  g and plasmid DNA was extracted from the cell pellets using the QIAprep<sup>®</sup> Spin Maxiprep Kit (Qiagen; Valencia, CA, USA) according to manufacturer's protocols.

#### 2.5.4. Site-Directed Mutagenesis

Site-directed mutagenesis of the pcDNA-p11 was performed with the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA.) using the primers designed by the QuikChange<sup>®</sup> Primer Design Program (<http://www.genomics.agilent.com/primerDesignProgram.jsp>). The primers used and the mutations they produced are detailed in Table 4. A PCR mixture was made according to Table 5. Next, the PCR reaction to produce the mutant vectors was 95°C for 30 seconds, followed by 14 cycles of 95°C for 30 seconds, 55°C for 60 seconds, 68°C for 400 seconds. Samples were kept on ice for two minutes. To digest the parental vector, 1 µL of Dpn I was added to each sample and incubated in a 37°C water bath for 1 hour. XL1 blue competent *E. coli* cells were thawed on ice and 50 µL of the *E. coli* was transformed using an empty vector control (pcDNA) or the pcDNA plasmids expressing p11-wildtype or p11 lysine-mutants listed in Table 3. The plasmids and XL1 blue *E. coli* were then incubated on ice for 30 minutes, heat-shocked at 42°C for 45 seconds, and then placed on ice for two minutes. Each transformant (25 µL) was spread across an LB-agar plate (containing 100 µg/mL AMP), using aseptic technique, and the plates were incubated at 37°C overnight.

**Table 4: List of p11 mutants and the primers used for site-directed mutagenesis.**

<b>Mutants of p11</b>	<b>Site-directed mutagenesis primer sets</b>
<b>P11 Lys54→Arg</b>	Fwd – 5'- CCA GGT CCT TCA TTA TTC TGT CCA CAG CCA GAG GG - 3'
	Rvs – 5'- CCC TCT GGC TGT GGA CAG AAT AAT GAA GGA CCT GG- 3'
<b>P11 Lys57→Arg</b>	Fwd – 5'- TGG TCC AGG TCC CTC ATT ATT TTG TCC ACA GCC AGA- 3'
	Rvs – 5'- TCT GGC TGT GGA CAA AAT AAT GAG GGA CCT GGA CCA- 3'

**Table 5: PCR mixture for site-directed mutagenesis.**

10X reaction buffer	5 $\mu$ L
dNTP mix	1 $\mu$ L
500 ng/ $\mu$ L plasmid	5 $\mu$ L
Primers (500 nM final concentration)	2.5 $\mu$ L of 10 $\mu$ M stock for forward and reverse
Deionized H <sub>2</sub> O	34 $\mu$ L
<b>FINAL VOLUME</b>	<b>50 <math>\mu</math>L</b>



## **2.6. Transfections**

### **2.6.1. Transient transfection**

HEK293T cells were seeded in 6-well plates ( $2.0 \times 10^5$  cells/well) and transfected the following day using the Lipofectamine2000™ transfection reagent (Invitrogen) in a serum-free OPTI-MEM medium (Invitrogen), according to manufacturer's protocols. First, culture medium was replaced with 1 mL of serum-free OPTI-MEM medium. Next, the transfection reagent mix was prepared from 14  $\mu$ l of the lipofectamine2000 transfection reagent and 56  $\mu$ L of serum-free OPTI-MEM (per well). A DNA dilution was then prepared from 1  $\mu$ g of plasmid DNA in 150  $\mu$ L of diluent (per well). Both the transfection reagent mix and the DNA dilution were incubated at room temperature for 5 min. The transfection reagent mix was then combined with the DNA dilution, incubated for another 5 min to form the transfection-DNA complexes. The transfection-DNA mixture was added to the cells with gentle swirling. The cells were subsequently incubated under standard culture conditions for 4 h. After this incubation, each well was supplemented with an equal volume of OPTI-MEM containing 10% FBS and cells were incubated for ~24 h.

### **2.6.2. Lentiviral-mediated stable transfection**

Cell lines depleted of p11 or p36 were produced using the pSUPER-retro plasmid containing shRNA.retro-scrambled control, shRNA.retro-p11, or shRNA.retro-p36. To produce lentiviral particles containing the pSUPER-retro plasmids, Phoenix-Ampho packaging cells ( $2 \times 10^5$  cells) were seeded in 25-cm<sup>3</sup> flasks and transfected with 4  $\mu$ g of the pSUPER-retro plasmids using the Lipofectamine2000™ transfection reagent (Invitrogen) according to manufacturer's protocol. Forty-eight hours post-transfection, the

target cell lines (NB4 and PR9) were infected by adding viral supernatants at a 1:1 ratio with fresh, complete RPMI-1640 media. Cells containing the viral supernatants were supplemented with 10  $\mu\text{g}/\text{mL}$  polybrene (Sigma). Forty-eight hours after infection, stable transfected cells were selected with 1  $\mu\text{g}/\text{mL}$  puromycin for  $\geq 1$  week.

## **2.7. Enzymatic Activity Assays**

### **2.7.1. Plasmin Generation Assay**

Suspended cells (NB4 and U937/PR9) or adherent MCF-7 cells were washed three times with Hanks Balanced salt solution (HBSS (Thermo-Fisher), containing 3 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$ , Thermo Fisher Scientific), and were seeded ( $1 \times 10^5$  cells) in 96-well plates. Adherent cells were plated overnight in 96-well plates and washed three times with HBSS before the assay. The cells were incubated at  $37^\circ\text{C}$  with and without 0.5  $\mu\text{M}$  glu-plasminogen for 20 – 30 minutes. This was followed by addition of 0.5 mM plasmin chromogenic substrate (Molecular Innovations, Novi, MI, USA). Plasmin activity was measured spectrophotometrically (At 405 nm, and 600 nm to account for turbidity) taking readings every 4 min for 4 h ( $n = 6$ ). Time course data were analyzed according to the equation describing the rate of p-nitroanilide (p-NA) production  $A_{405 \text{ nm}} = B + Kt^2$ , where K is the rate constant for the acceleration of p-NA generation and B is the y-intercept. Under these experimental parameters, K is proportional to the initial rate of the plasmin produced from plasminogen.

### **2.7.2. Tissue Factor Activity Assay**

NB4 cells (maintained at a cell density of  $0.3 \times 10^6$  cells/mL) were treated with or without 1  $\mu$ M ATRA for 48 h, washed once using 1X DPBS, lysed in RIPA lysis buffer, and incubated on ice for 5 min. Cell lysates were centrifuged at 14,800 x g for 10 min at 4°C and tissue factor (TF) activity of samples was assessed using the Tissue Factor Human Chromogenic Activity Assay Kit (Abcam) according to the manufacturer's protocol. Briefly, total cell extracts (60  $\mu$ g of protein in a 10  $\mu$ L volume) were combined with a mixture of the assay buffer, Factor VII and Factor X. Samples were seeded in a 96-well plate and incubated for 30 minutes at 37°C. The chromogenic Factor Xa substrate was added and absorbance was determined at 405 nm using a plate reader spectrophotometer (Molecular Devices; SpectraMax M3, Sunnyvale, CA, USA). Readings were obtained every 5 min for 25 min.

### **2.7.3. Proteasome Activity Assay**

NB4 cells ( $\sim 0.3 \times 10^6$  cells/mL) were treated with or without ATRA for 48 h. Cells were then washed once with 1X DPBS, lysed in RIPA lysis buffer, and incubated on ice for 5 min. Cell lysates were centrifuged at 14,800 x g for 10 min at 4°C. Cell extracts (10  $\mu$ g) were incubated with 50  $\mu$ mol/L fluorogenic peptide substrate Suc-LLVY-AMC (Enzo Life Sciences) in 200  $\mu$ L assay buffer [25 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.05% NP-40, and 0.001% SDS (w/v)] for 1 h at 37°C. The fluorescence determination was carried out at 380 nm for excitation and 440 nm for emission using a plate reader spectrophotometer (Molecular Devices; SpectraMax M3).

## **2.8. Nitroblue Tetrazolium (NBT) Assay**

NB4 or NB4-MR2 cells were seeded in a 6-well dish (100,000 cells/ 5mL) and treated several hours later using 1  $\mu$ M ATRA or DMSO as a vehicle control. Cells were treated with DMSO or ATRA again the following day. After five days, 250,000 cells were centrifuged at 300 x g for 5 min and cell pellets were resuspended in 1X DPBS and centrifuged once again. The cell pellet was resuspended in nitroblue tetrazolium (NBT) solution [0.2% 4-nitroblue tetrazolium, 2  $\mu$ g/mL PMA in a volume of 1.5 mL of RPMI-1640 medium] and incubated at 37°C for 15 min. Next, cells were washed with three times with cold 1X DPBS and the cell pellet wash resuspended in 200  $\mu$ L of 1X DPBS after the final wash. Cells that stained positive and negative with NBT were counted using a haemocytometer and the percentage of NBT positive-stained cells was represented as %NBT-positive = (NBT-positive cells / Total cell number) x 100.

## **2.9. Protein Purification**

### **2.9.1. Purification of Recombinant p11**

The pAED4.91-S100A10 construct was transformed into BL21(DE3) pLysS competent *E.coli*, expressed and purified according to Ayala-Sanmartin *et al.*<sup>265</sup> with modifications. Bacterial cell pellets were lysed by sonication (60 s for 3 pulses) or by French press (1000 psi). Lysis was performed in 100 mM imidazole (pH 7.5), 400 mM NaCl, 10 mM MgCl<sub>2</sub>, 2 mM DTT (Dithiothreitol) with inhibitors and centrifuged at 30,000 x g for 40 min. The supernatant was precipitated with 50% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and centrifuged at 27,000 x g for 20 min. The resulting supernatant was applied to a Butyl-Sepharose column (GE Healthcare Life Sciences, Piscataway NJ USA)) that was equilibrated with the same

buffer. S100A10 was eluted with a linear gradient from 50% to 0%  $(\text{NH}_4)_2\text{SO}_4$  in the same buffer. The eluate was dialyzed against 40 mM Tris, pH 7.4, 150 mM NaCl, 0.5 mM EGTA, 0.5 mM DTT and subjected to gel permeation chromatography on a HiLoad 16/600 Superdex 75 (GE) column. S100A10 eluted as a single peak on gel permeation chromatography.

### **2.9.2. Purification of Recombinant p36**

The pAED4.91-Annexin A2 construct was transformed into BL21(DE3) pLysS competent *E.coli*, and expressed and purified according to Khanna *et al.*<sup>266</sup> with modifications. Bacterial cell pellets were lysed by French press (1000 psi). Lysis was performed in 20 mM imidazole pH 7.5, 150 mM NaCl, 5 mM EGTA 3 mM DTT with inhibitors and centrifugation at 30,000 x g for 40 min. The NaCl concentration was reduced to 50 mM, prior to loading the supernatant onto a DEAE (MacroPrep DE, BioRad, Hercules, CA, USA) equilibrated in 20 mM Imidazole pH 7.5, 25 mM NaCl 1mM DTT. Next, 10 mM phosphate was added to the flow through fraction and applied to a CHT™ Ceramic Hydroxyapatite (BioRad) equilibrated in 10 mM potassium phosphate pH 7.0. Annexin A2 was eluted with a linear gradient from 0.01 to 1 M KPi pH 7.0. The eluate was dialyzed against 40 mM Tris, pH 7.4, 150 mM NaCl, 0.5 mM EGTA, 0.5 mM DTT and subjected to gel permeation chromatography on a HiLoad 16/600 Superdex 75 (GE) column.

### **2.9.3. Purification of Human Recombinant Annexin A2 Heterotetramer (AII<sub>t</sub>)**

Equimolar amounts of purified (gel filtered) recombinant human S100A10 and recombinant human Annexin A2 were mixed together and incubated on ice for 30 min.

The resulting complex was gel filtered on a HighLoad™ 16/600 Superdex 200 pg Size Exclusion column (GE Healthcare Life Sciences) equilibrated with 40 mM Tris-HCl pH 7.4 150 mM NaCl 0.5 mM EGTA 0.5 mM DTT. The resulting elution profile shows a major peak consisting of recombinant human annexin A2 heterotetramer (rhAII<sub>t</sub>), followed by minor peaks of annexin A2 and S100A10 monomers. Peak identify was confirmed by SDS-PAGE. Positively identified fractions were pooled and concentrated. Protein content determined by absorbance at 280 nm, using an extinction co-efficient 1 mg/mL of 0.68.

#### **2.10. *In vitro* Proteasomal Degradation Assay**

The ability of the 20S proteasome to degrade purified recombinant proteins was assessed using the 20S proteasome assay kit (Boston Biochemical, Boston, MA) according to manufacturer's protocols. Briefly, 'reaction buffer' was diluted to 1X and the 3% SDS 'proteasome activation' solution was added to the buffer at a final concentration of 0.03%. Samples were prepared in this buffer with or without 1 µg of purified 20S proteasome alone or in combination with 250 µM lactacystin (reconstituted in deionized water [dH<sub>2</sub>O]). Next, 1 µg of purified recombinant human proteins ( p11, p36, or AII<sub>t</sub> proteins) were added to the mixture at a final volume of 20 µL and incubated for 1 h at 37°C. The reaction was stopped by the addition of 20 µL of 2X sample loading buffer, and then boiled in water for 5 min. The protein lysate was resolved by SDS-PAGE and analyzed by western blot analysis.

### **2.11. Immunoprecipitation**

For immunoprecipitation, cells were lysed in cell lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% NP-40, 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM EDTA, and complete EDTA-free protease and inhibitor cocktail (Thermo) and 200 µg of precleared cell lysates were incubated with antibodies for mouse IgG1, IgG2A (R&D, Minneapolis, MN, USA), p36 (a gift from Dr. Tony Hunter<sup>267</sup>), p11 (BD), or phospho-AKT (ser473; Cell Signaling) for 1 h at 4°C. Afterwards, the lysates were incubated using protein G-agarose or protein A agarose (Santa Cruz) or protein G-sepharose (Abcam) beads for 1 h at 4°C to collect immune complexes (antibody bound to the target protein). The beads were washed four times in cell lysis buffer, and the immune complexes were eluted from the beads by addition of 40 µL 2X SDS sample buffer and incubation at 50°C for 10 min. The supernatants of the eluted samples were incubated in boiling water for 5 min and then used for western blot analysis.

### **2.12. Mass spectrometry analysis**

After the immunoprecipitation, p11 protein were separated by SDS-PAGE and stained with coomassie blue. The bands were excised from the gel and digested with trypsin for mass spectrometry analysis according to Shevchenko *et al.*, 2006<sup>268</sup>, with some modifications. Briefly, the gel bands were reduced with 0.5 M dithiothreitol and alkylated with 0.7 M iodoacetamide. Gel bands were digested with trypsin (Promega, Madison, WI) for 12 h at 37°C. Peptides were extracted from the gel bands with 100 µL of a 50% acetonitrile, 5% formic acid solution. The extract was dried by vacuum centrifugation (SPD SpeedVac, Thermo Electron Corp. Waltham, MA) and the tryptic peptides were resuspended in 20 µL of a 3% acetonitrile, 0.5% formic acid solution. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using a

nano flow liquid chromatography system (Ultimate3000, ThermoScientific) interfaced to a hybrid ion trap/orbitrap high-resolution tandem mass spectrometer (VelosPro, Thermo Scientific) operated in data-dependent acquisition (DDA) mode. Briefly, 1  $\mu$ L of each sample was injected onto a capillary column (4  $\mu$ m Jupiter C18 manually packed on a 30 cm x 75 $\mu$ m ID PicoFrit Column, New Objective) at a flow rate of 300 nL/min. Samples were electro-sprayed at 1.2 kV using a dynamic nanospray ionization source. Chromatographic separation was carried out using 90-minute linear gradients (Mobile Phase A: 0.1% formic acid in MS-grade water, mobile phase B: 0.1% formic acid in MS-grade acetonitrile) from 3% B to 35% B over 60 min, then increasing to 95% B over five min. MS/MS spectra were acquired using both collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) for the top 15 peaks in the survey 30000-resolution MS scan. The raw files were acquired (Xcalibur, Thermo Fisher) and exported to Proteome Discoverer 2.0 (ThermoFisher) software for peptide and protein identification using SequestHT search algorithm (Parameters: full trypsin digestion with 2 maximum missed cleavages, 10 ppm precursor mass tolerance and 0.8 Da fragment mass tolerance). Database searching was done using the UniprotKB human database.

## **2.13. Protein Expression Analysis**

### **2.13.1. Preparation of Protein Lysates**

Cells were washed with cold 1X DPBS and lysed in cold, complete RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25% sodium deoxycholate, 1% triton X-100. The following were added upon use: 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM EDTA, and complete EDTA-free protease and phosphatase inhibitor cocktails (Thermo)].



For lysing adherent cells, cells were washed once in 5 mL of 1X DPBS and lysed directly on the culture plate, which was on ice. The cell lysates were collected from the plate using a cell scraper and transferred to microcentrifuge tubes. For lysing suspended cell cultures, cells were collected from culture flasks, centrifuged for 5 min at 500 x g at room temperature. The cell pellets were resuspended in 5 mL of 1X DPBS and centrifuged again (5 min at 500 x g, room temperature). After washing, the cell pellets were lysed in 50 -100  $\mu$ L of complete RIPA lysis buffer while on ice, and total cell lysates transferred to microcentrifuge tubes. Total cell lysates were incubated on ice for 10 min and centrifuged at 14, 800 x g for 10 min at 4°C.

### **2.13.2. SDS-PAGE and Western blot Analysis**

Protein of total cell lysates (20-40  $\mu$ g for cell lysates) was loaded into each well and resolved by SDS-PAGE on 10-20% (or 5% for PML/RAR $\alpha$  western blots) acrylamide gel and the proteins were transferred onto nitrocellulose membranes. Antibody complexes were viewed on the Odyssey IR imaging system (LI-COR Biosciences). Protein expression was quantified using ImageJ software (NIH).

## **2.14. Quantitative (real-time) Polymerase Chain Reaction (qPCR)**

### **2.14.1. Total RNA isolation and cDNA synthesis**

The phenol-based RIBOzol RNA extraction reagent (Amresco, Dallas, TX, USA) was used to extract RNA from cells according to manufacturer's instructions. Briefly, cells were lysed using 1 mL of RIBOzol and transferred to an Eppendorf tube. Next, 200  $\mu$ L of chloroform was added to the mixture, shaken vigorously, and incubated at room temperature for 5 min. The mixture was centrifuged at 12, 000 x g for 10 min at 4°C and

then the aqueous phase was collected and used to purify total RNA using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocols. Total RNA concentration was determined by using a 1:100 dilution in ddH<sub>2</sub>O, and measured using the Eppendorf BioSpectrometer (Eppendorf, Mississauga, ON). Purity of total RNA was determined by the A<sub>260</sub>/A<sub>280</sub> ratio. The cDNA was directly synthesized from total RNA (1 µg) using the QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's instructions.

#### **2.14.2. qPCR Primers, Reaction and Analysis**

Starting with 25 ng of cDNA, the reaction as carried out using the SSO Advanced Universal SYBR Green Supermix (BioRad) and the CFX96 Real-Time PCR Detection System (Bio-Rad) to amplify the genes of interest using the primers (final concentration of 0.5 µM; IDT, Coralville, IA, USA) detailed in Table 6. Fold change values were calculated using the  $\Delta\Delta C_t$  method<sup>269</sup> and normalized to  $\beta$ -actin, GAPDH, and H RTP1 expression. An unpaired *t*-test was used to calculate statistical significance.

**Table 6: List of genes with their forward (Fwd; 5' to 3') and reverse (Rvs; 5' to 3') primers used for qPCR analysis.**

<b>Target Gene &amp; Reference Sequence</b>	<b>Primer Set</b>	<b>Amplicon Size (bp)</b>	<b>Primer Efficiency</b>
<b>P11 (NM_002966.2)</b>	Fwd – GGA CCA GTG TAG AGA TGG CA	150	98.1%
	Rvs – TTA TCA GGG AGG AGC GAA CT		
<b>P36 (NM_001002858.2)</b>	Fwd – CAA GAC CAA AGG TGT GGA TG	124	112.1%
	Rvs – CAG TGC TGA TGC AAG TTC CT		
<b>GAPDH (NM_002046.5)</b>	Fwd – TCA AGA AGG TGG TGA AGC AG	93	104.7%
	Rvs – CGC TGT TGA AGT CAG AGG AG		
<b>HPRT1 (NM_000194.2)</b>	Fwd – TTG CTT TCC TTG GTC AGG CA	116	104.9 %
	Rvs – ATC CAA CAC TTC GTG GGG TC		
<b>β-actin (NM_001101.3)</b>	Fwd – ACG TTG CTA TCC AGG CTG TG	85	100.7%
	Rvs – GAG GGC ATA CCC CTC GTA GA		

## **2.15. *In silico* Analysis**

### **2.15.1. Predictions of Intrinsic Disorder in p11**

Search for intrinsically disordered domains of p11 was performed using the a well-characterized online disorder predictor program PONDR VL-XT.14<sup>270,271</sup>. The PONDR VL-XT.14 program integrated three different neural networks (one for N-terminal regions, a second for internal regions and a third for C-terminal regions.) and was trained using experimentally confirmed disordered protein regions. The VL-XT training set included disordered segments of 40 or more amino acid residues as characterized by X-ray and NMR for the predictor of the internal regions and segments of five or more amino acid residues for the predictors of the two terminal regions.

### **2.15.2 Determination of Surface Exposure of Residues in p11**

The online program GETAREA<sup>272</sup> was used to calculate the surface exposure. GETAREA calculates the surface exposure of each amino acid as a ratio of its exposed surface area in the crystal structure. Residues were scored as surface exposed, if the ratio value exceeds 50%

### **2.15.3. Identification of a Direct Repeat Separated by 5bp (DR5) Retinoic Acid Receptor Response Element (RARE) in S100A10 Promoter Region**

A single protein-coding transcript variant was identified within the ENSEMBL (GRCh38.p10) database for human S100A10 (ENSG00000197747; S100A10-202) at Chr1:151,982,915-151,994,390 [reverse strand]; with the promoter region predicted to be located at Chr1:151,992,200-151,994,201. The FASTA sequence  $\pm 10$  kb from the transcription start site (Chr1:151,944,390) was examined using the MEME-suite4.12.0

Motif –based sequence analysis tools FIMO (Finding Individual Motif occurrences) and Tomtom (compares one or more motifs against a database of known motifs)<sup>273</sup>. Using FIMO, the TSS  $\pm$ 10 kb region was scanned for the classical RARE, which is a repeated hexameric motif of RGKTSA separated by 5 nucleotides (DR5) [RGKTSANNNNRGKTSA] as well as the lesser understood DR1-4 RAREs<sup>274</sup>. A putative DR5 [AGGTGAGGCCAGGCTCA] was identified in the promoter region and compared against a database of known transcription factor binding motifs in MEME-suite (Tomtom; searched against Jolma2013 and JASPAR CORE 2014 vertebrates) to confirm the motif's affinity for retinoic acid receptors

## **2.16. Statistical Analysis**

Data are expressed as the mean  $\pm$  S.D. of the indicated number of samples. Statistical significance was determined by Student t-test for unpaired observations or 1-way ANOVA with Tukey multiple comparisons, where  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  and  $****P < 0.0001$  were considered statistically significant.

**CHAPTER 3: RESULTS - MECHANISMS REGULATING P11 EXPRESSION IN  
ACUTE PROMYELOCYTIC LEUKEMIA (APL) AND ALL-TRANS RETINOIC  
ACID (ATRA) THERAPY**

**3.1 Regulation of p11 and p36 by the PML/RAR $\alpha$  oncoprotein and ATRA treatment**

**3.1.1. ATRA downregulates p11 and p36 in NB4 cells and NB4 cells resistant to ATRA-induced differentiation**

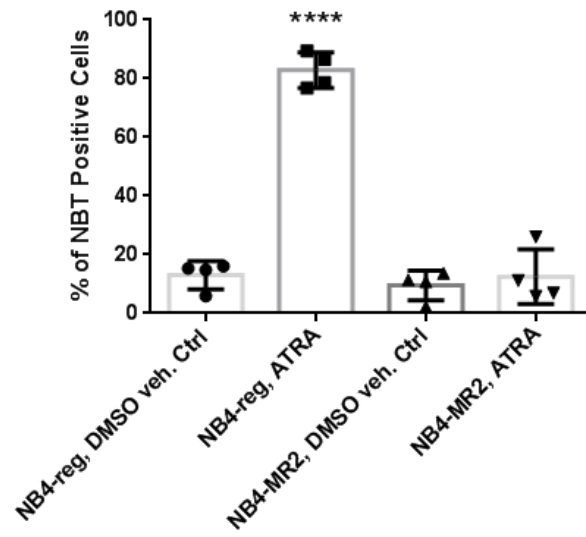
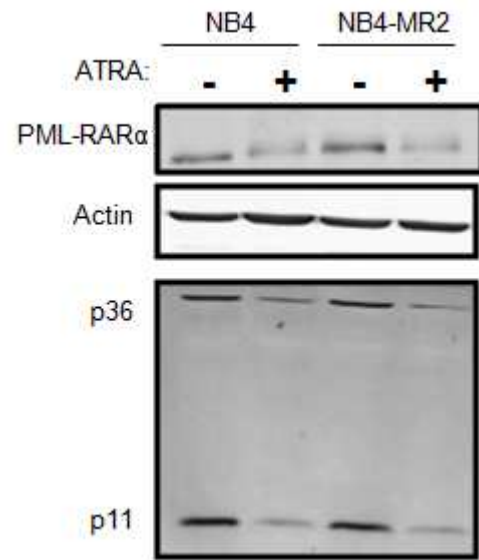
All-trans retinoic acid (ATRA) treatment induces the granulocytic differentiation and degradation of PML/RAR $\alpha$  in the NB4 cells, a patient-derived APL promyelocyte cell line, but it is not certain whether granulocytic differentiation or the loss of PML/RAR $\alpha$  are responsible for the downregulation of p11 and p36 expression in ATRA-treated NB4 cells. Accordingly, the effect of ATRA on p11 and p36 expression was examined using a NB4 cell line resistant to ATRA-induced differentiation (NB4-MR2)<sup>275</sup>. To confirm that the NB4-MR2 cells were resistant to ATRA-induced differentiation, granulocytic differentiation was assessed using the nitroblue tetrazolium blue (NBT) assay. After five days of ATRA treatment, 78.10 $\pm$ 9.3% of ATRA-treated NB4 cells stained positive whereas only 12.45 $\pm$ 9.32% of ATRA-treated NB4-MR2 cells stained with NBT (Figure 10A), similar to the untreated NB4 and NB4-MR2 cells. Therefore, this confirmed that ATRA was incapable of inducing differentiation of the NB4-MR2 cells. O'Connell *et al*<sup>125</sup> previously showed that ATRA treatment of NB4 cells resulted in the downregulation of p11 and p36 after 24 and 48 h, respectively. However, it is uncertain whether this ATRA induced the downregulation of p11 and p36 through inducing the degradation of PML/RAR $\alpha$  or by inducing differentiation of the NB4 cells. Hence, PML/RAR $\alpha$ , p11 and p36 expression was assessed in the NB4 and NB4-MR2 cells to

determine if differentiation contributed to the loss of p11 and p36 caused by ATRA treatment. ATRA treatment (72 h) of both NB4 cell lines produced a loss of PML/RAR $\alpha$ , p11 and p36 expression (Figure 10B), indicating that p11 and p36 expressions are regulated by PML/RAR $\alpha$  and not affected by cell differentiation. PML/RAR $\alpha$  expression was examined after 48 h of ATRA treatment, but no change occurred in PML/RAR $\alpha$  levels (Data not shown), yet p11 and p36 expression were dramatically reduced at this time (see Section 3.2.1.). These results indicate that the downregulation of p11 and p36 by ATRA treatment is likely a result of its effect on PML/RAR $\alpha$  and not differentiation.

**Figure 10: ATRA down-regulates p11, p36 and PML/RAR $\alpha$  expression in NB4 cells and NB4-MR2 cells, which are resistant to ATRA-induced granulocytic differentiation.**

(A) NBT reduction assay on NB4 or NB4-MR2 cells after 5-day ATRA treatment. Data is expressed as the mean  $\pm$  S.D. of four independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*\*\*\*P < 0.0001 is considered statistically significant. (B) Cell lysates were prepared from NB4 or NB4-MR2 were treated without or with 1  $\mu$ M ATRA for 72 h, and the levels of the indicated proteins were examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as three independent experiments.



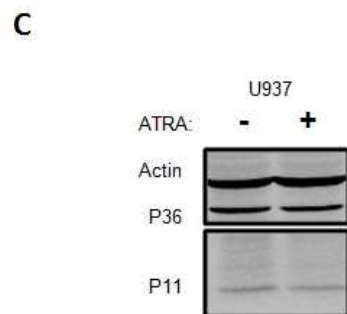
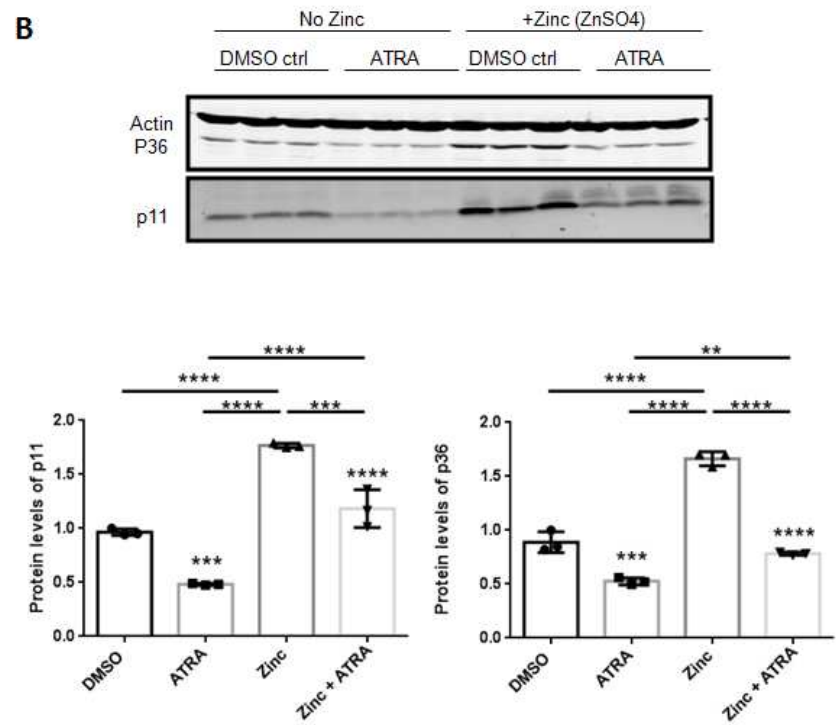
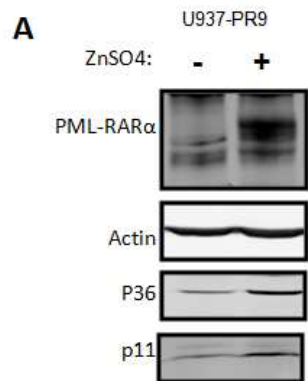
**A****B**

### **3.1.2. ATRA reduces p11 and p36 expression in U937/PR9 lymphoma cells with and without PML/RAR $\alpha$ induction**

We observed that ATRA downregulation of PML/RAR $\alpha$  correlates with the loss of p11 and p36, and therefore inducing PML/RAR $\alpha$  expression should increase p11 and p36. The effect of PML/RAR $\alpha$  induction and ATRA treatment was assessed using the U937/PR9 (PR9) cell line that expresses PML/RAR $\alpha$  on a zinc-inducible promoter. Consistent with findings by O'Connell *et al.*<sup>125</sup>, induction of PML/RAR $\alpha$  expression in PR9 cells resulted in the upregulation of p11 and p36 protein levels (Figure 11A) concomitant with the induction of PML/RAR $\alpha$ . Since we observed that ATRA induces the loss of p11, p36, and PML/RAR $\alpha$  in NB4 cells, we expect that ATRA would cause a loss of p11 and p36 expression in PML/RAR $\alpha$ -induced PR9 cells. ATRA treatment reduced p11 and p36 expression in non-induced (by  $2.0 \pm 0.008$ -fold ( $P < 0.0001$ ) and  $1.69 \pm 0.03$ -fold, ( $P < 0.001$ ) respectively) and in PML-induced PR9 cells (by  $1.49 \pm 0.01$ -fold,  $P < 0.0001$  and  $2.04 \pm 0.001$ -fold,  $P < 0.001$ , respectively) (Figure 11B). ATRA treatment did not affect the expression of p11 or p36 in U937 cells (Figure 11C), the parent cell line of the PR9 cell line<sup>276</sup>, suggesting that the ATRA-induced loss of p11 and p36 in the non-induced PR9 cells may be due to 'leaky' expression of PML/RAR $\alpha$  from the zinc-inducible vector. We also observed that p36 levels returned to basal levels in zinc-induced p36 knockdown cells making it difficult to determine if PML/RAR $\alpha$  can increase p11 levels independent of PML/RAR $\alpha$ -induced increases in p36.

**Figure 11: PML/RAR $\alpha$  is a positive regulator of p11 and p36 expression in PR9 cells.**

Cell lysates were prepared from (A) PR9 cells treated with or without 100  $\mu$ M zinc sulfate (ZnSO<sub>4</sub>) for 48 h, (B) ATRA-treated (1  $\mu$ M for 48 h) PR9 cells ( $\pm$ 100  $\mu$ M ZnSO<sub>4</sub>) for 48 h and (C) ATRA-treated (1  $\mu$ M for 48 h) U937 cells. Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*\*P < 0.01, \*\*\*P < 0.001 and \*\*\*\*P < 0.0001 are considered statistically significant.

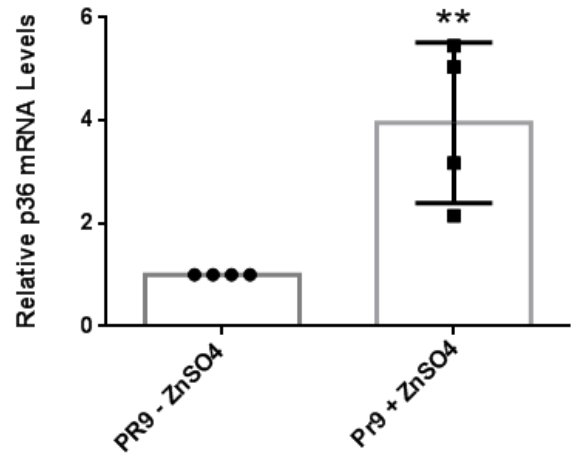
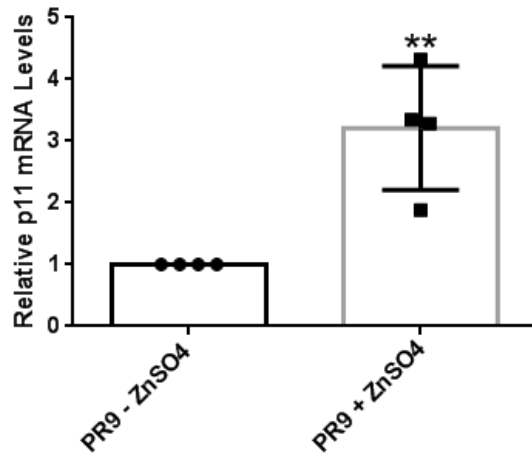


### **3.1.3. Induction of PML/RAR $\alpha$ upregulates p11 and p36 transcript levels**

ATRA was shown to induce the degradation of PML/RAR $\alpha$  concomitant with the loss of p11 and p36 protein levels without affecting mRNA levels<sup>125</sup>. In addition, we have previously shown that induction of PML/RAR $\alpha$  expression in PR9 cells resulted in the upregulation of p11 and p36 at the protein level without affecting mRNA levels. These findings showed that PML/RAR $\alpha$  regulated p11 and p36 expression at the post-translational levels, but not the transcriptional level. However, other studies contradicted these finding by demonstrating that p11 mRNA levels were unaffected<sup>125</sup> or reduced<sup>277</sup> in ATRA-treated NB4 cells compared to the control group. Hence, we re-examined p11 and p36 mRNA levels to determine whether they were affected by the induction of PML/RAR $\alpha$  expression using PR9 cells. The induction of PML/RAR $\alpha$  in PR9 cells resulted in the increase of p11 and p36 mRNA levels by  $3.65 \pm 0.58$  ( $P < 0.01$ ) and  $3.95 \pm 1.56$ -fold ( $P < 0.01$ ) respectively (Figure 12), indicating a direct regulation of these genes by PML/RAR $\alpha$ .

**Figure 12: Induction of PML/RAR $\alpha$  in PR9 cells up-regulates p11 and p36 transcript levels.**

Total RNA extracted from PR9 cells treated (48 h) without or with 100  $\mu$ M ZnSO<sub>4</sub>, was used for cDNA synthesis. The relative expression of p11 and p36 mRNA levels was determined from cDNA (25 ng) by qPCR analysis and normalized to GAPDH,  $\beta$ -actin and HPRT1. Data is expressed as the mean  $\pm$  S.D. of four independent experiments. Statistical significance was determined using the Student t-test for unpaired observations, where \*\*P < 0.01 is considered statistically significance.



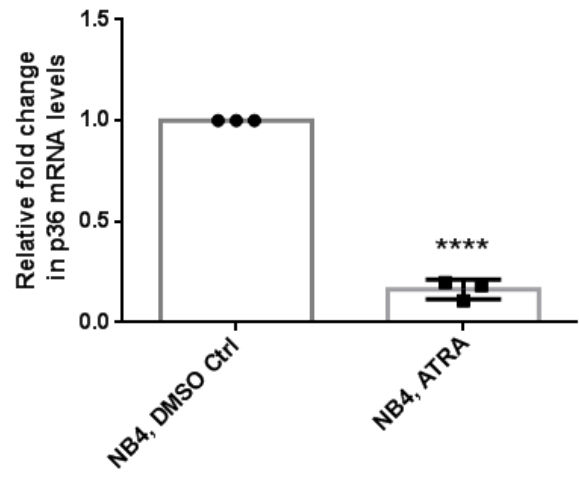
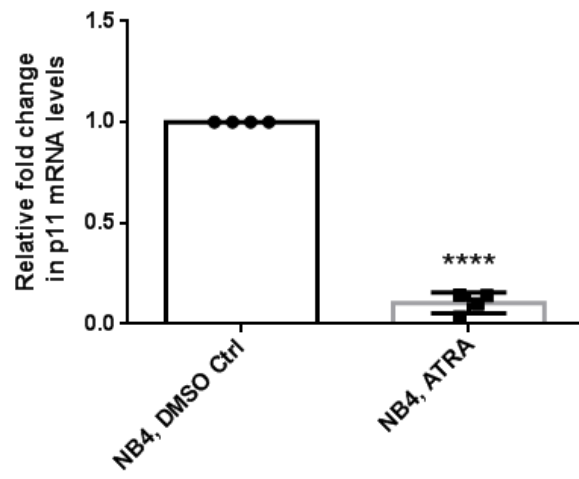
### **3.1.4 ATRA treatment downregulates p11 and p36 transcript levels in NB4 cells**

ATRA treatment of NB4 cells decreased p11 protein, but the mRNA levels were shown to be unaffected<sup>125</sup> or reduced<sup>277</sup>. However, we showed that induction of PML/RAR $\alpha$  expression upregulated p11 and p36 mRNA levels. Since induction of PML/RAR $\alpha$  expression increases p11 and p36 mRNA levels, we would expect that depletion of PML/RAR $\alpha$  by ATRA treatment would decrease p11 and p36 mRNA if PML/RAR $\alpha$  does directly regulate mRNA levels of both p11 and p36. To clarify the effect of ATRA, the mRNA levels of p11 and p36 in ATRA-treated NB4 cells were assessed by qPCR. ATRA treatment (48 h) of NB4 cells significantly reduced p11 and p36 mRNA levels by 10.0 $\pm$ 0.05-fold ( $P < 0.0001$ ) and 6.25 $\pm$ 0.05-fold ( $P < 0.0001$ ) respectively compared to the control group (Figure 13). This downregulation of p11 and p36 mRNA levels by ATRA treatment was concomitant with the downregulation of protein levels of p11 and p36 at 48 h (See section 3.2.1.). This demonstrates that ATRA-induced loss of PML/RAR $\alpha$  mediates the decrease of p11 and p36 expression at the transcriptional and protein levels.



**Figure 13: ATRA down-regulates p11 and p36 transcript levels in NB4 cells.**

Total RNA extracted from NB4 cells treated (48 h) without or with 1  $\mu$ M ATRA was used for cDNA synthesis. The relative expression of p11 and p36 mRNA levels was determined from cDNA (25 ng) by qPCR analysis and normalized to GAPDH,  $\beta$ -actin and HPRT1. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using the Student t-test for unpaired observations, where \*\*\*\*P < 0.0001 is considered statistically significance.



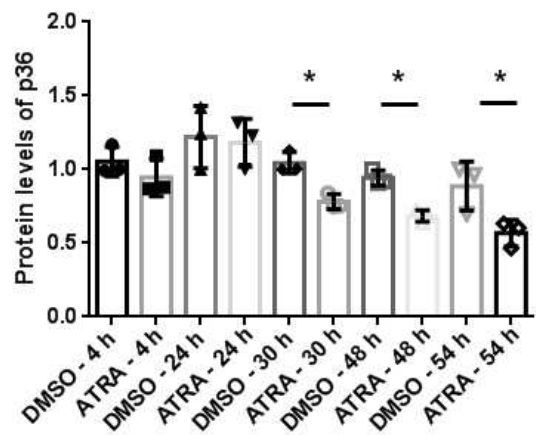
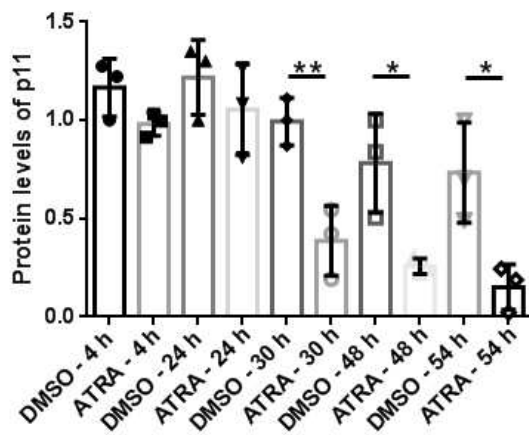
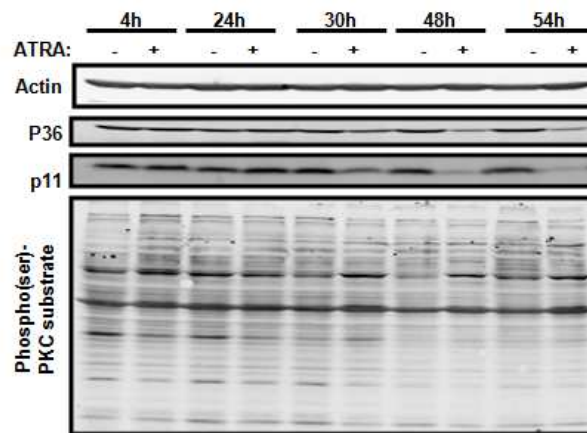
## **3.2. ATRA treatment downregulates p11 and p36 independent of ATRA-stimulated Protein Kinase C (PKC) activity or p36 serine-phosphorylation**

### **3.2.1. ATRA downregulation of p11 and p36 protein levels correlates with increased PKC activation in NB4 cells**

Protein kinase C (PKC)-dependent phosphorylation of p36 on Ser11 and Ser25 is hypothesized to prevent the interaction with p11, and promotes p11 proteasomal degradation<sup>83,90</sup>. Since ATRA activates several isoforms of PKC<sup>129,278</sup>, the relationship between the ATRA-induced p11 and p36 loss and PKC activity was examined in NB4 cells. Similar to our previous findings, p11 and p36 protein levels were dramatically reduced by  $2.56 \pm 0.17$ -fold ( $P < 0.01$ ) and  $1.35 \pm 0.05$ -fold ( $P < 0.05$ ) respectively after 30 h of ATRA treatment. PKC activity was assessed by western blotting using an antibody for detection of phosphoserine PKC substrates<sup>280</sup>, which detects substrates of PKC that are phosphorylated on serine residues. As expected, the levels of phosphoserine PKC substrates increased at 30 h of ATRA treatment compared to control treatments (Figure 14). This indicates that ATRA induces PKC activity, which could disrupt the interaction between p11 and p36 through the PKC-dependent phosphorylation of p36.

**Figure 14: ATRA treatment of NB4 cells induces PKC activity concomitant with the loss of p11 and p36 expression.**

NB4 cells were treated with 1  $\mu$ M ATRA or a vehicle control for the indicated times. Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using the Student t-test for unpaired observations, where \*P < 0.01, and \*\*\*\*P < 0.0001 are considered statistically significant. The Student t-test was performed for each day in which p11 or p36 expression of ATRA-treated NB4 cells was compared to the control group.

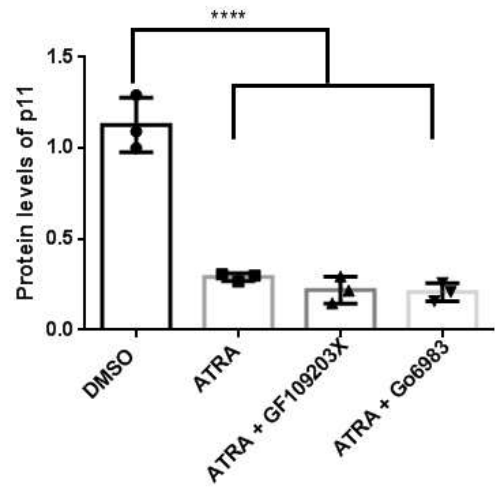
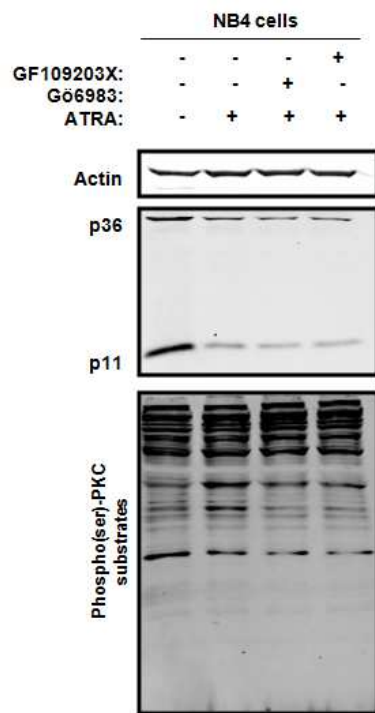


### **3.2.2. Inhibition of PKC activity does not prevent the ATRA-induced loss of p11 or p36 in NB4 cells.**

The contribution of ATRA-induced PKC activity to the loss of p11 and p36 was examined using NB4 cells. The cells were treated with ATRA alone or in combination with the PKC inhibitors: GF109203X (IC<sub>50</sub> for PKC- $\alpha$ , - $\beta$ , - $\delta$  is 20 nM, 16-17 nM, and 20 nM respectively<sup>281</sup>) and Gö6983 (IC<sub>50</sub> for PKC- $\alpha$ , - $\beta$ , - $\delta$ , - $\gamma$ , and - $\zeta$  is 7 nM, 7 nM, 10 nM, 6 nM, and 60 nM respectively<sup>282</sup>). If PKC-dependent phosphorylation of p36 or a p36-independent PKC activity contributes to the loss of p11 and p36 in ATRA-treated NB4 cells, then inhibition of PKC should prevent the loss of p11 and/or p36. Neither GF109203X nor Gö6983 prevented the ATRA-induced downregulation of p11 or p36 in NB4 cells. To confirm the inhibition of PKC activity, phosphoserine-PKC substrate levels were assessed. As expected, the presence of GF109203X and Gö6983 reduced the ATRA-induced increase in phosphoserine-PKC substrate (Figure 15). This indicates that although ATRA induced PKC activity, PKC may not be involved in the ATRA induced loss of p11 and p36 expression in NB4 cells.

**Figure 15: Inhibition of PKC activity does not prevent the ATRA-induced loss of p11 and p36 expression in NB4 cells.**

NB4 cells were treated with 1 $\mu$ M ATRA alone or in combination with 1  $\mu$ M of GF109203X or Gö6983 for 48 h. Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*\*\*\*P < 0.0001 is considered statistically significant.



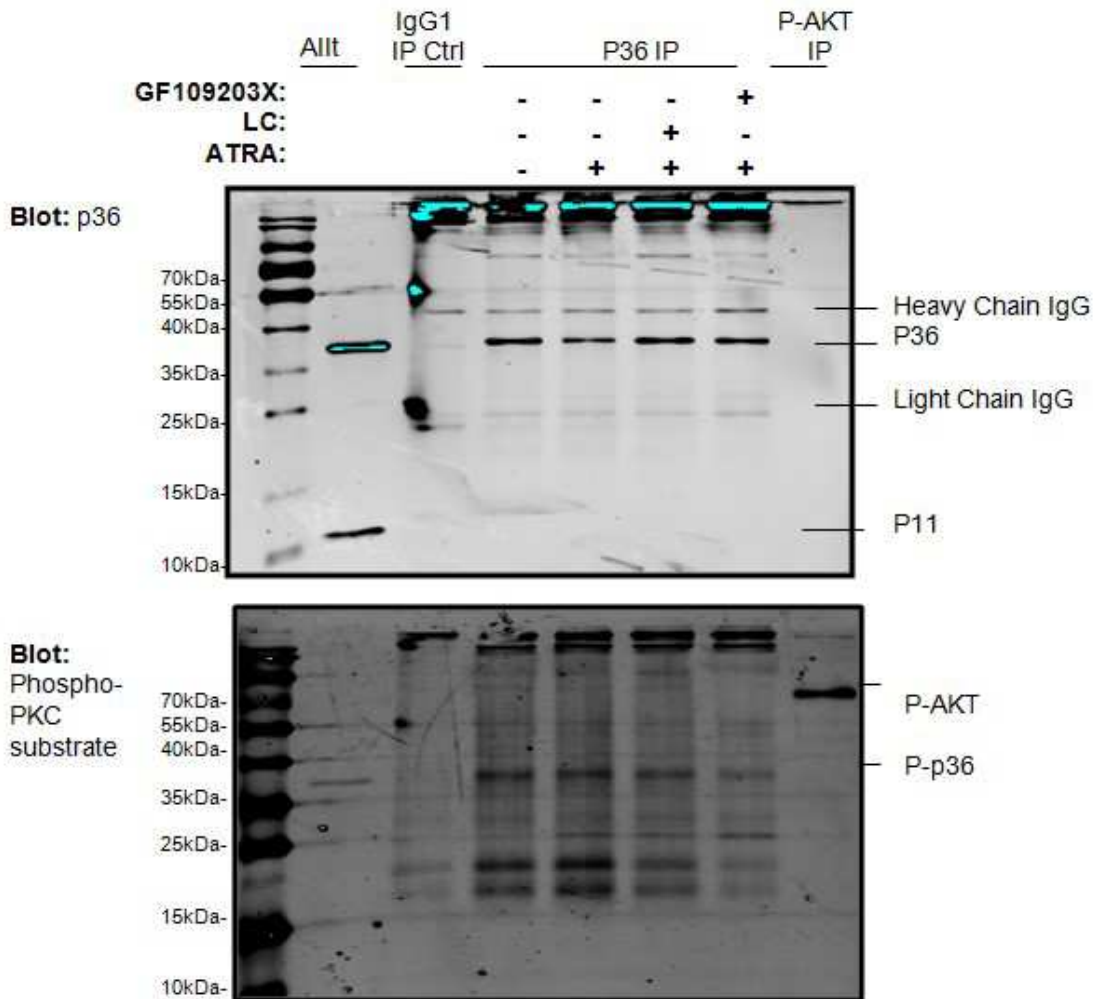


### **3.2.3. Serine-phosphorylated p36 levels are not affected by ATRA in NB4 cells**

To determine if ATRA-induced PKC activity caused serine-phosphorylation of p36, we examined the phosphorylation of p36 by immunoprecipitation. P36 and phospho-AKT (serine473; positive control) were immunoprecipitated from NB4 cells treated with or without ATRA for 24 h. Samples were obtained at 24 h rather than 48 h or later time points due to the loss of p36 occurring after 30 h of ATRA treatment. Western blots for phosphoserine detected phospho-Akt, and phosphorylated p36 under all conditions (Figure 16). The level of phospho-p36 was decreased in NB4 cells treated with ATRA and GF109203X, indicating that inhibition of the PKC  $\alpha$  and  $\beta$  isoforms contributed to p36 phosphorylation. In addition, the inhibition of PKC activity appeared to partially block p36 phosphorylation. Although, diminishing the levels of phosphorylated p36 would be expected to allow the non-phosphorylated p36 to bind to p11 and prevent p11 degradation, we observed in Figure 15 that PKC inhibition did not prevent the ATRA-induced loss of p11 expression in NB4 cells. This indicates that blocking the formation of the p11-p36 heterotetramer complex by PKC-phosphorylation of p36 may not occur in ATRA-treated NB4 cells even though ATRA stimulated PKC activity.

**Figure 16: ATRA treatment does not affect serine-phosphorylation of p36 in NB4 cells.**

Immunoprecipitation of p36, phospho-AKT (serine473), or IgG2A isotype control of NB4 cells treated for 24 h with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M lactacystin or 1  $\mu$ M of GF109203X. Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis. Data is expressed as three independent experiments.



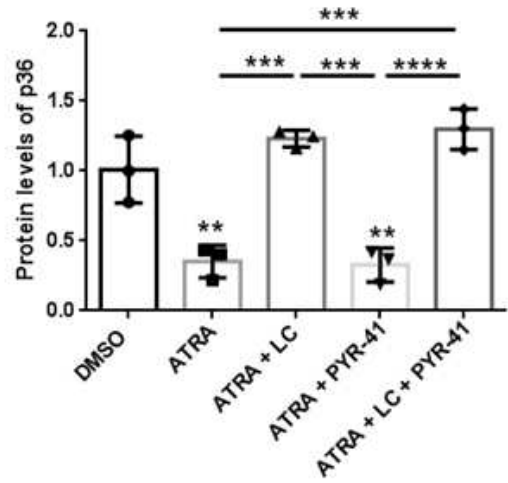
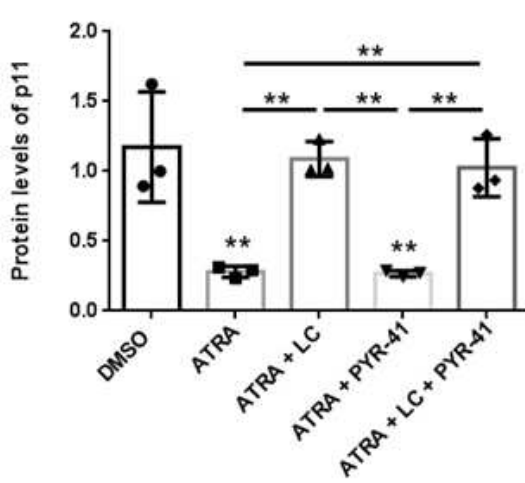
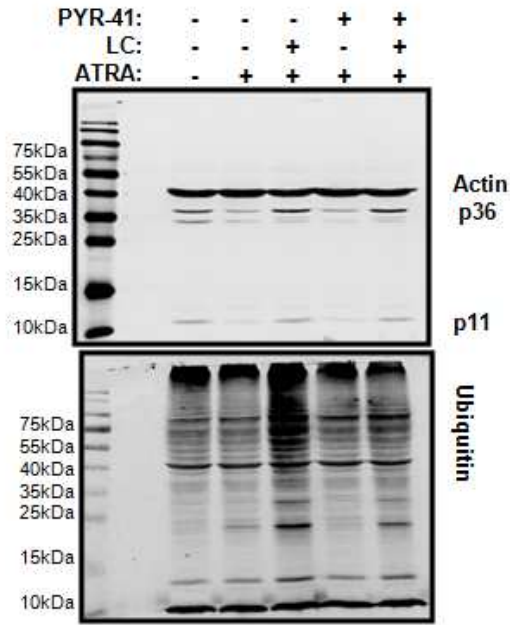
### **3.3. ATRA induces proteasomal degradation of p11 independent of its ubiquitylation**

#### **3.3.1. Inhibition of proteasomal activity, but not ubiquitylation, prevents the ATRA-induced loss of p11 in NB4 cells**

Central to our understanding of the regulation of p11, it has been reported that agents which downregulated p36 resulted in the rapid ubiquitylation and proteasomal degradation of the unpartnered p11<sup>83,90</sup>. This study and our previous study<sup>125</sup> demonstrated that the ATRA treatment of NB4 cells caused a dramatic loss of p11 and p36 expression. To re-examine if ATRA downregulates p11 protein levels by ubiquitylation and proteasomal degradation, NB4 cells were treated for 48 h with ATRA alone or in combination with the proteasome inhibitor lactacystin (LC), the E1-ubiquitylation enzyme inhibitor PYR-41 or both. Western blot analysis of ATRA-treated NB4 cells revealed a significant downregulation of p11 and p36 expression of  $3.57 \pm 0.04$ -fold ( $P < 0.01$ ) and  $2.86 \pm 0.11$ -fold ( $P < 0.01$ ) respectively (Figure 17). The presence of PYR-41 did not prevent the ATRA-dependent loss of p11; however, LC reversed the ATRA-dependent loss in p11. NB4 cells treated with ATRA and LC showed increased levels of ubiquitin-conjugated proteins confirming that LC blocked proteasomal degradation, resulting in the accumulation of ubiquitylated proteins. Furthermore, the addition of PYR-41 to cells treated with ATRA and LC prevented the accumulation of ubiquitin-conjugated proteins observed with ATRA and LC treatment, confirming that PYR-41 inhibited the ubiquitylation of proteins. Unexpectedly, higher molecular weight species of p11 were not found in NB4 cells treated with ATRA and LC, indicating that p11 was not ubiquitylated under these conditions.

**Figure 17: Inhibition of proteasomal degradation, but not ubiquitylation, prevents the ATRA-induced loss of p11 and p36 in NB4 cells.**

NB4 cells were treated for 48 h with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M lactacystin (LC) or 2  $\mu$ M PYR-41. Cell lysates were prepared and the level of the indicated proteins were examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*\*P < 0.01, \*\*\*P < 0.001, and \*\*\*\*P < 0.0001 are considered statistically significant.



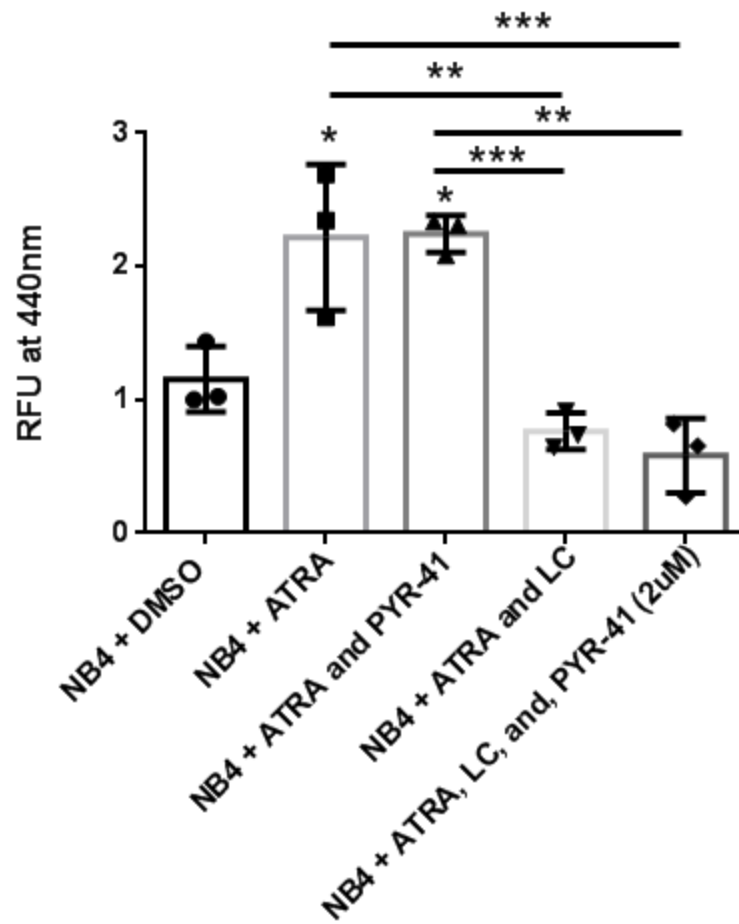
### 3.3.2. Proteasomal activity is increased in ATRA-treated NB4 cells

Proteasome activity was shown to be upregulated in ATRA-treated NB4 cells without affecting the levels of the 20S proteasome core<sup>283</sup>. This increase in proteasomal activity could contribute to the loss of p11 observed in ATRA-treated NB4 cells. Hence, the effect of ATRA on the proteasome activity was assessed using NB4 cells treated in the presence of ATRA alone or in combination with LC, PYR-41, or both. Proteasomal activity was determined in cell lysates by the ability of proteasome to cleave the fluorogenic proteasome substrate Suc-LLVY-AMC. Cell lysates were examined after 48 h of treatment as p11 and p36 protein levels are dramatically downregulated at this time point (See section 3.2.1.). ATRA-treated NB4 cells showed a  $1.92 \pm 0.55$ -fold ( $P < 0.05$ ) increase in proteasome activity compared to the DMSO control treatments, and the presence of lactacystin prevented the ATRA-induced proteasome activity in these cells (Figure 18). PYR-41 did not affect proteasome activity as expected. This indicates that ATRA-stimulated proteasomal activity may be a contributing factor to the loss of p11 and p36 protein levels observed in ATRA-treated NB4 cells.

**Figure 18: ATRA induces proteasomal activity in NB4 cells.**

NB4 cells were treated for 48 h with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M LC, 2  $\mu$ M PYR-41. Cell lysates were prepared and 10  $\mu$ g of protein was incubated with 50  $\mu$ mol/L fluorogenic peptide substrate Suc-LLVY-AMC in 200  $\mu$ L assay buffer [25 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.05% NP-40, and 0.001% SDS (w/v)] for 1 h at 37°C. Fluorescence determination was carried out at 380 nm for excitation and 440 nm. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 are considered statistically significant.





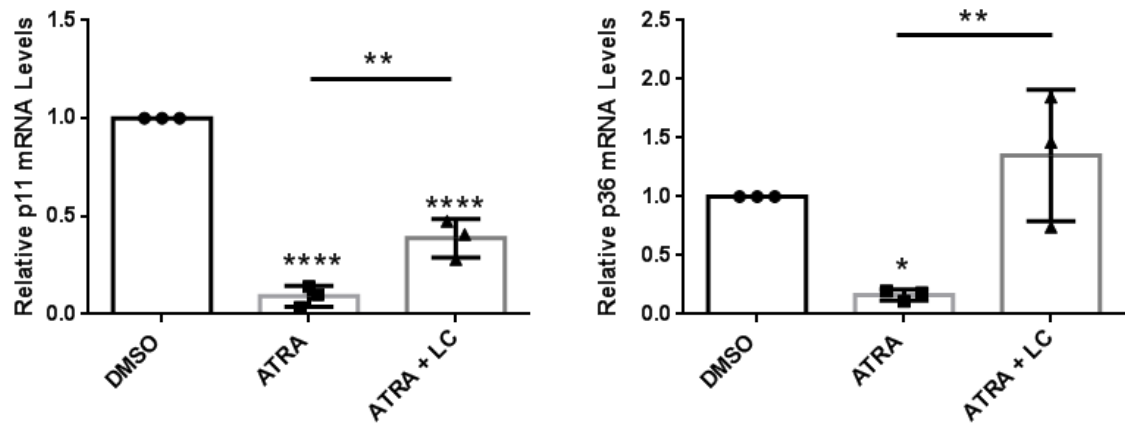
### **3.3.3. LC treatment upregulates p11 and p36 transcript levels in NB4 cells**

Because ATRA and LC treatment upregulated p11 and p36 protein levels, we examined if LC contributed to the upregulation of p11 and p36 at the transcriptional level. The presence of LC and ATRA increased p11 and p36 transcript levels compared to ATRA alone, but p11 expression was significant lower than that of the control group by  $2.78 \pm 0.05$ -fold ( $P < 0.0001$ ), and p36 was not significantly increased (Figure 19A). LC alone did not cause a significant increase of p11 or p36 transcript levels compared to the control group (Figure 19B).

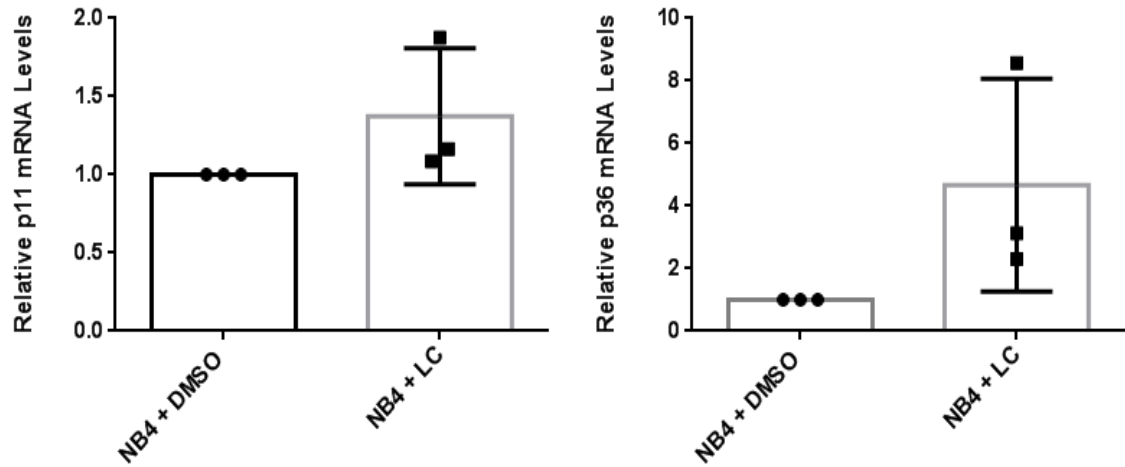
**Figure 19: Treatment of NB4 cells with LC alone or in combination with ATRA upregulates p11 and p36 transcript.**

Total RNA extracted from NB4 cells treated (48 h) without or with (A) 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M LC or (B) 2  $\mu$ M LC alone were used for cDNA synthesis. The relative expression of p11 and p36 mRNA levels was determined from cDNA (25 ng) by qPCR analysis and normalized to GAPDH,  $\beta$ -actin and HPRT1. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using (A) one-way ANOVA (with Tukey multiple comparisons) or (B) the Student t-test for unpaired observations, where \*P < 0.05, \*\*P < 0.01, and \*\*\*\*P < 0.0001 are considered statistically significance.

A



B

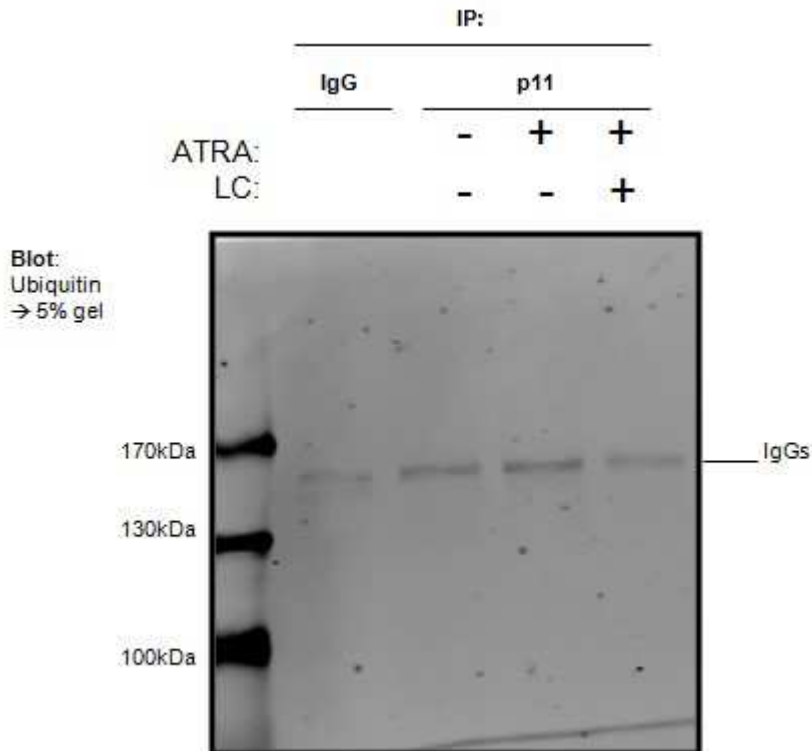
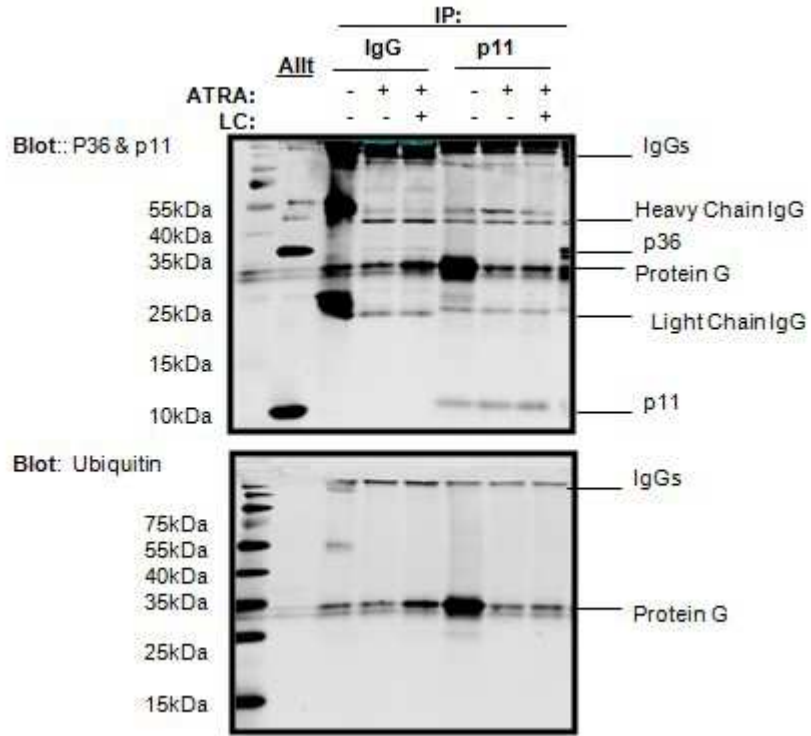


#### **3.3.4. ATRA treatment does not induce ubiquitylation of p11 in NB4 cells**

Our observation that the ATRA-induced loss of p11 and p36 was not prevented by PYR-41 indicated that ubiquitylation was not required for p11 proteasomal degradation. To explore this further, NB4 cells were treated with ATRA alone or in combination with LC, and p11 was immunoprecipitated and analyzed by western blotting for ubiquitin. Interestingly, no ubiquitin-conjugates of p11 were detected even when cells were treated with ATRA and LC (Figure 20A), indicating that ATRA did not induce p11 ubiquitylation. Analysis of these western blots with anti-p11 antibody failed to identify any higher molecular weight species of p11 even using 5% percentage acrylamide gels (Figure 20B).

**Figure 20: Ubiquitylated p11 is not detected in NB4 cells treated with ATRA alone or in combination with LC.**

Immunoprecipitation of p11 or IgG2a isotype control from cell lysates (200  $\mu$ g) of NB4 cells treated for 24 h with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M LC. The p11 or IgG2A control immunoprecipitates were eluted from protein agarose G or A beads and supernatants were boiled for 5 min. The indicated proteins were examined by western blot analysis. Data is expressed as three independent experiments.



### **3.3.5. Ubiquitylated p11 is absent in p36-depleted PR9 cells**

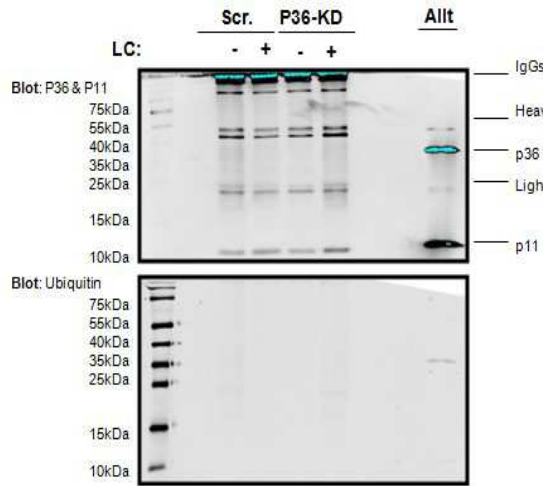
According to the prevailing dogma, the interaction between p36 and p11 prevents the ubiquitylation of p11<sup>90,92</sup>. Therefore, we reasoned that if we immunoprecipitated p11 from p36-depleted cells and probed western blots for ubiquitin, then LC-dependent inhibition of the proteasome would result in the accumulation of ubiquitylated p11. As expected, p36-depletion resulted in a loss of p11 expression, and LC treatment of the p36-depleted cells restored p11 protein levels; however, ubiquitin-conjugated p11 was not detected in LC-treated p36-depleted cells (Figure 21A, upper panel). Furthermore, no higher molecular weight forms of p11 were detected using lower percentage acrylamide gels (5%; Figure 21, lower panel). The absence of higher molecular weight species of p11 and our inability to detect any ubiquitin conjugates of p11 strongly indicated that the loss of p11 protein expression observed with p36-depleted cells was due to ubiquitin-independent proteasomal degradation. We also immunoprecipitated p11 from purified AII to determine if p11 immunoprecipitation would isolate the p11 protein bound to p36 protein. Immunoprecipitation of p11 from purified AII did isolate the p11 protein, but there was a low amount of p36 associated with p11 (Figure 21B).



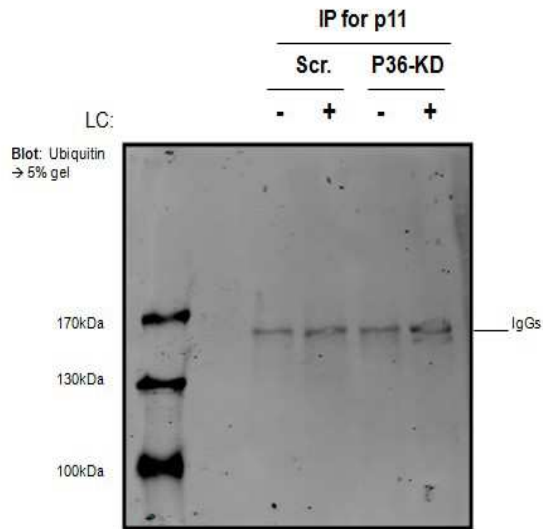
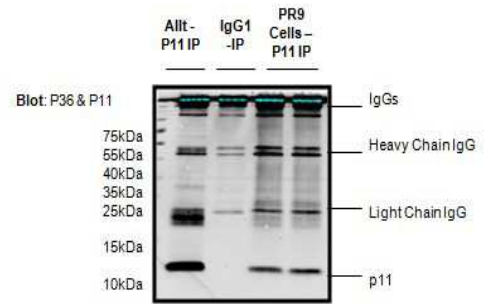
**Figure 21: Ubiquitylated p11 is not detected in p36-depleted PR9 cells with or without LC treatment.**

Immunoprecipitation of p11 or IgG1 isotype control from cell lysates (200 µg) of (A) PR9 cells treated for 48 h with 2 µM LC and (B) PR9 cells and purified AII<sub>t</sub> heterotetramer protein (the p11<sub>2</sub>-p36<sub>2</sub> complex; 0.25 µg used for immunoprecipitation of p11) isolated from bovine lung. The p11 or IgG1 control immunoprecipitates were eluted from protein agarose G beads and supernatants were boiled for 5 min. The indicated proteins were examined by western blot analysis. Data is expressed as three independent experiments.

A



B

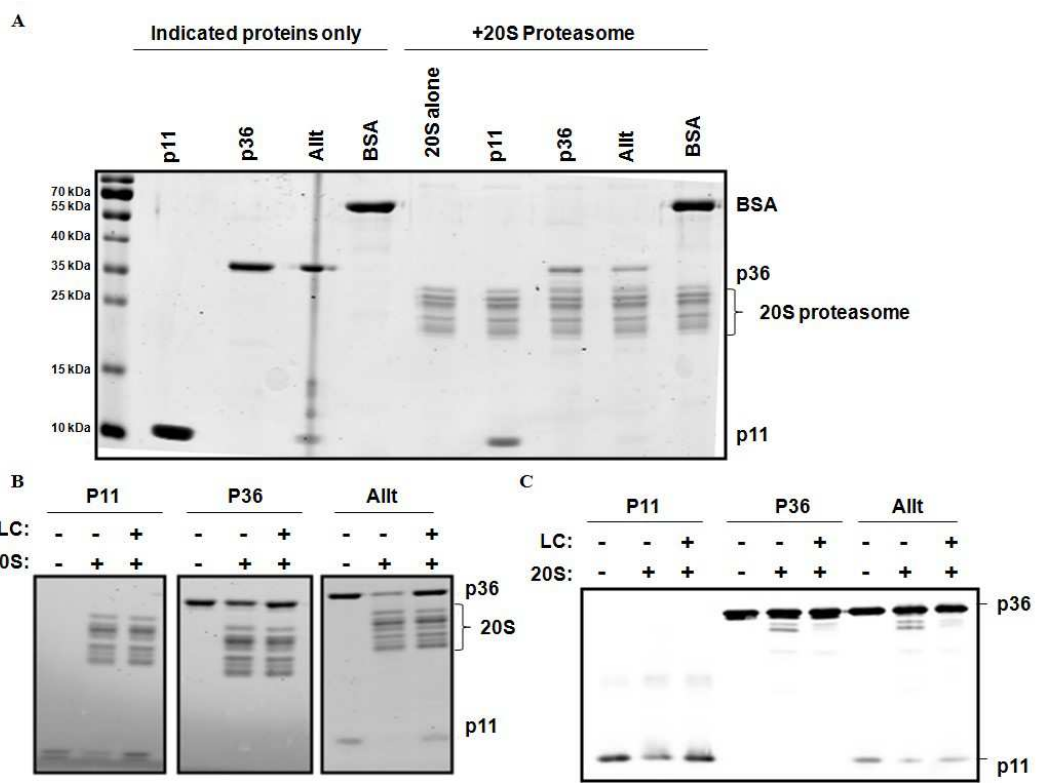


### **3.3.6. P11, p36 and AIIIt are degraded by the 20S proteasome**

Ubiquitin-dependent proteasomal degradation is mediated by the 26S proteasome complex that consists of the 20S core and the 19S cap, which is present on one of the ends of the 20S core complex (Reviewed in <sup>234</sup>). Yet certain proteins are directly degraded by the 20S proteasome independent of ubiquitylation. To investigate if p11 was a substrate for ubiquitin-independent degradation by the 20S proteasome, purified p11, p36 or AIIIt proteins were incubated in the presence of a purified 20S proteasome preparation. We observed that p11 and p36 were degraded by the 20S proteasome (Figure 22A-C), and this was blocked by LC *in vitro* (Figure 22B-C). Bovine serum albumin (BSA), a negative control, was not proteolyzed under thesis conditions (Figure 2A). Together, these data support that proteasomal degradation of p11 can be regulated in an ubiquitin-independent manner.

**Figure 22: The 20S proteasome degrades purified p11, p36, and the p11-p36 heterotetramer, AIIIt in an ubiquitin-independent manner *in vitro*.**

(A) Purified p11 (1  $\mu$ g), p36 (1  $\mu$ g), bovine AIIIt heterotetramer (1  $\mu$ g), and bovine serum albumin (1  $\mu$ g BSA; negative control) were incubated for 1 h at 37°C in the absence or presence of the 20S proteasome (1  $\mu$ g) in buffer [25 mM HEPES, 0.05 mM EDTA, pH 7.6] containing 0.03% SDS for proteasomal activation. (B, C) Purified p11 (1  $\mu$ g), p36 (1  $\mu$ g), bovine AIIIt heterotetramer (1  $\mu$ g) protein were incubated for 1 h at 37°C in the absence or presence of the 20S proteasome (1  $\mu$ g) in buffer containing 0.03% SDS for proteasomal activation with or without LC (0.5 mM). Samples were subjected to SDS-PAGE and gels were examined by (A, B) staining overnight using coomassie brilliant blue or (C) by western blot analysis. Data is expressed as three independent experiments. Victoria Miller produced purified proteins and Ryan Holloway performed the assays and western blot analysis.

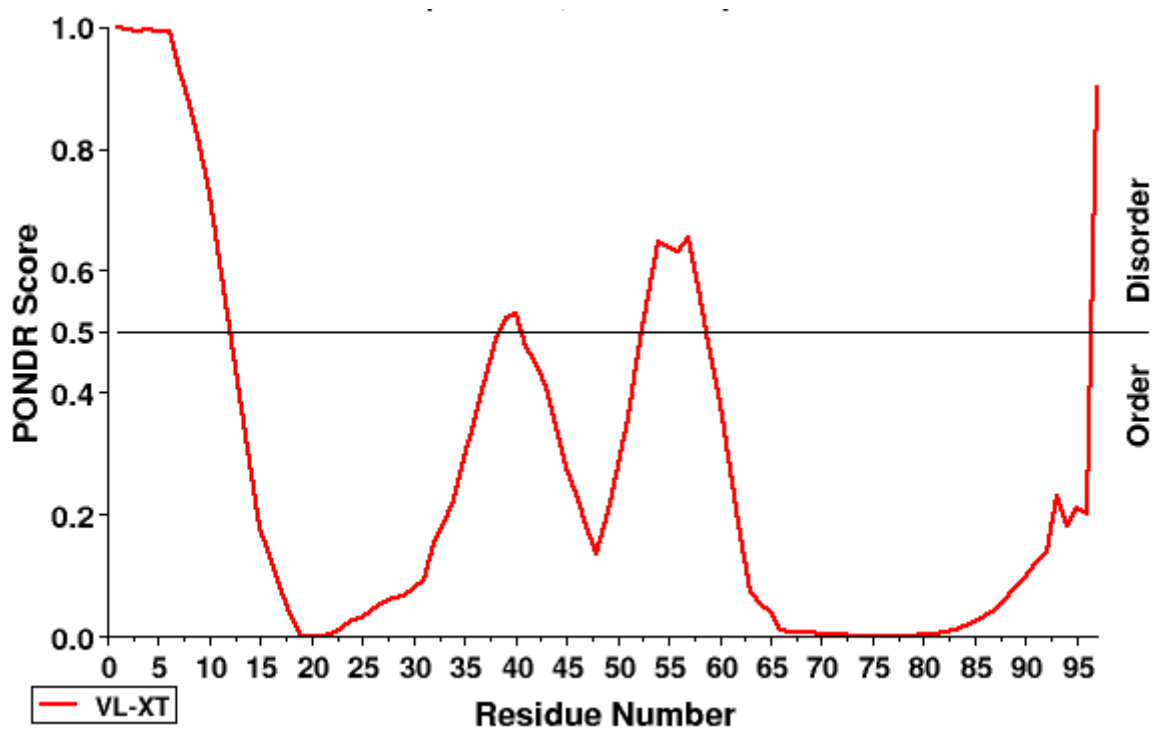


### **3.3.7. Intrinsic disordered regions predicted in p11 amino acid sequence**

A key feature of proteins degraded by the 20S proteasome in an ubiquitin-independent manner is the presence of intrinsic disordered regions. Since we observed that non-ubiquitylated p11 was degraded by the 20S proteasome *in vitro*, this suggested that p11 contains intrinsic disordered regions. To identify unstructured regions of the p11 protein, the amino acid sequence of p11 was analyzed using the PONDR online program, which identifies unstructured regions of proteins based on information obtained from x-ray crystallography and mass spectrometry databases. The PONDR program predicted that the p11 protein has disordered regions located on its carboxy- and amino-terminal regions, and at ~Lys38-40 and ~Lys52-60 (Figure 23). The prediction that p11 contains intrinsic disordered regions supports the fact that p11 can be a substrate of the 20S proteasome and p11 can be degraded in an ubiquitin-independent manner.

**Figure 23: Intrinsic disordered regions predicted from the p11 amino acid sequence using the online program, PONDR**

Plot of the PONDR VL-XT predicted disorder for p11 (residues 1–97) comparing the PONDR score, which indicates the % of disorder, to the corresponding amino acid residue of p11.





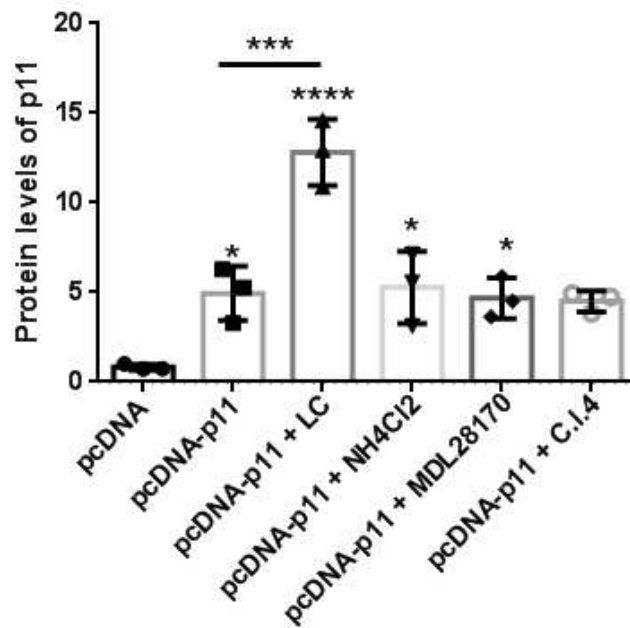
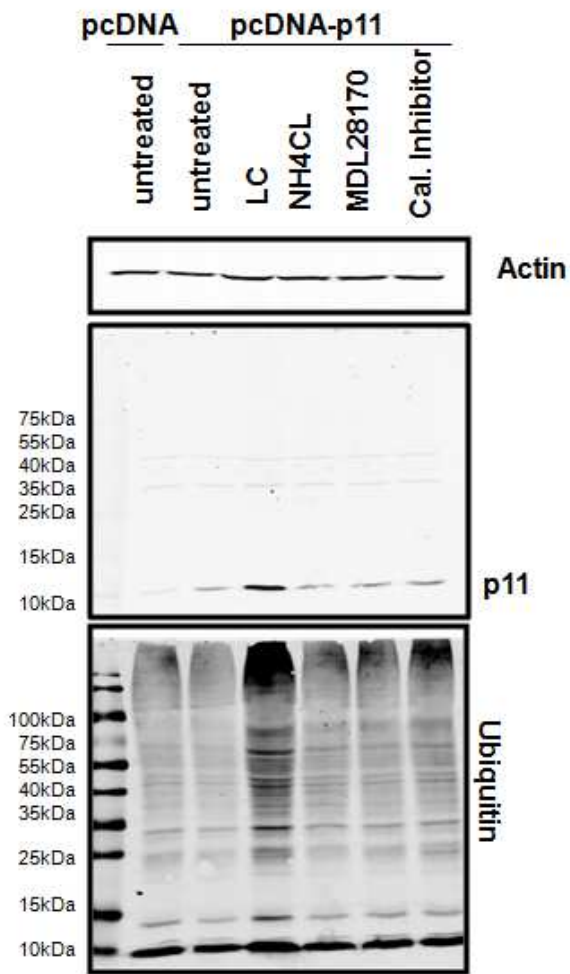
### **3.4. P11 can be ubiquitylated on Lys57, but may not contribute to its proteasomal degradation**

#### **3.4.1. Proteasomal inhibition increases p11 expression in HEK293T cells transiently overexpressing p11**

In order to examine the possibility that other types of proteolytic regulatory mechanisms might regulate p11 levels, HEK293T cells were transfected with a p11-expressing construct and treated with proteolytic inhibitors. Since p36 and p11 are expressed at low levels in HEK293T cells, we reasoned that the forced expression of p11 in the presence of low intracellular levels of p36 would result in the accumulation of p11 only if the appropriate proteolytic regulatory pathway could be inhibited. We utilized inhibitors of lysosomal and calpain proteolytic pathways and observed that p11 accumulated only in the presence of LC, indicating that the proteasomal regulatory pathway is a key pathway for regulation of p11 levels (Figure 24).

**Figure 24: Proteasome inhibition upregulates p11 protein levels in p11-overexpressing HEK293T cells.**

HEK293T cells were transiently transfected in 6-well plates using 2.5 µg/well of pcDNA3.1-empty vector or -p11 vector alone. The next day, cells were treated for 18 h with or without 3 µM lactacystin (LC), 1 mM NH<sub>4</sub>Cl, 40 µM MDL28170, or 50 µM calpain inhibitor IV (C.I.4)). Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with β-actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean ± S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*P < 0.05, \*\*\*P < 0.001, and \*\*\*\*P < 0.0001 are considered statistically significance.



### **3.4.2. P11 is multi-ubiquitylated in HEK293T cells co-expressing p11 and wildtype or lysine-less ubiquitin, and p36 inhibits p11 ubiquitylation.**

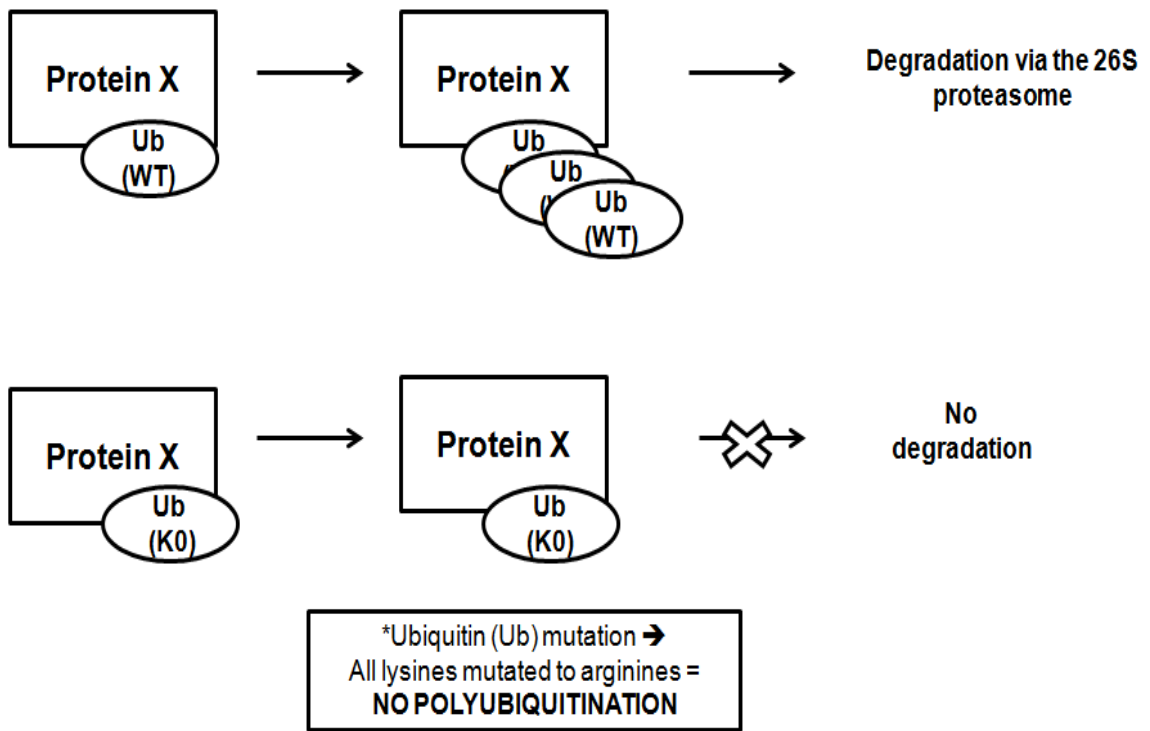
P11 has been shown to be ubiquitylated when co-expressed with ubiquitin in HEK293T cells<sup>90,92</sup>. Hence, p11 was transiently co-expressed with wildtype ubiquitin (ub-WT) or a mutant ubiquitin in which all lysine residues were mutated to arginine (ub-K0) (Figure 25) to prevent the formation of polyubiquitin chains and consequently ubiquitin-mediated proteasomal degradation of target proteins. Here, a low amount of p11-expression vector was transfected into the cells and the co-expression of ub-K0 should prevent p11 from being polyubiquitylated and directed to the proteasome for degradation. Compared to the control group, HEK293T cells overexpressing p11 alone or with ub-WT expressed did not show a significant increase in p11 expression. However, co-expression of p11 and ub-K0 resulted in  $4.0 \pm 0.45$ -fold ( $P < 0.001$ ) more p11 than control. Furthermore, higher molecular weight forms of p11, which also corresponded to the molecular weights of ubiquitylated p11 (i.e. 19.5 kDA, 28 kDA, etc), were detected only when cells were transfected with both p11 and ub-WT or ub-K0 (Figure 26A), although the latter was more dramatic. The presence of multiple molecular weight species of p11 in cells transfected with ub-K0 demonstrated the presence of multiple ubiquitin sites on p11.

The prevailing hypothesis is that ubiquitylation and 26S proteasomal degradation of p11 is prevented by the protein-protein interaction between p11 and p36. We observed that higher molecular weight forms of p11 are produced when p11 and ubiquitin are co-expressed in HEK293T cells, which express p36 protein at a low level. The ubiquitylation of p11 likely occurs due to an insufficient presence of p36 protein that would inhibit p11 ubiquitylation. If the prevailing hypothesis is correct, expressing p36

with p11 and ubiquitin in HEK293T cells should prevent ubiquitylation of p11. Compared to the control group, HEK293T cells overexpressing p11 and p36 showed a significant increase in p11 expression (Figure 26B). HEK293T cells overexpressing p11 and p36 also showed a significant increase in p11 expression compared to cells expressing p11 and ubiquitin-K0. However, no higher molecular weight forms of p11 were observed in HEK293T cells that co-expressed p11, p36 and ub-K0 as compared to HEK293T cells that co-expressed p11 and ub-K0. These results show that p11 is ubiquitylated when there is low levels of p36 protein, and that higher molecular weight forms of p11 are absent when p11 and ub-K0 are co-expressed with p36. This supports the prevailing hypothesis that p36 prevents p11 ubiquitylation, which could direct p11 to the 26S proteasome for degradation and therefore promote the stability of the p11 protein.

**Figure 25: Model illustrating the expected effects of wildtype ubiquitin (ub-WT) versus a mutant ubiquitin with all of the lysines changed to arginine residues (ub-K0).**

Wildtype-ubiquitin (ub-WT) allows for the ubiquitylation of substrate protein on lysine residues, followed by the attachment of other ubiquitin proteins to the ubiquitin attached to the substrate protein. This formation of a chain of multiple ubiquitin units, or polyubiquitylation, directs protein to the 26S proteasome for degradation. The lysineless mutant of ubiquitin (ub-K0) is a mutated form of ubiquitin engineered to have all of the lysine residues in ubiquitin substituted with arginine. The absence of lysine residues in the ub-K0 mutant consequently inhibits the formation of polyubiquitin chains that would direct protein to the 26S proteasome for degradation.

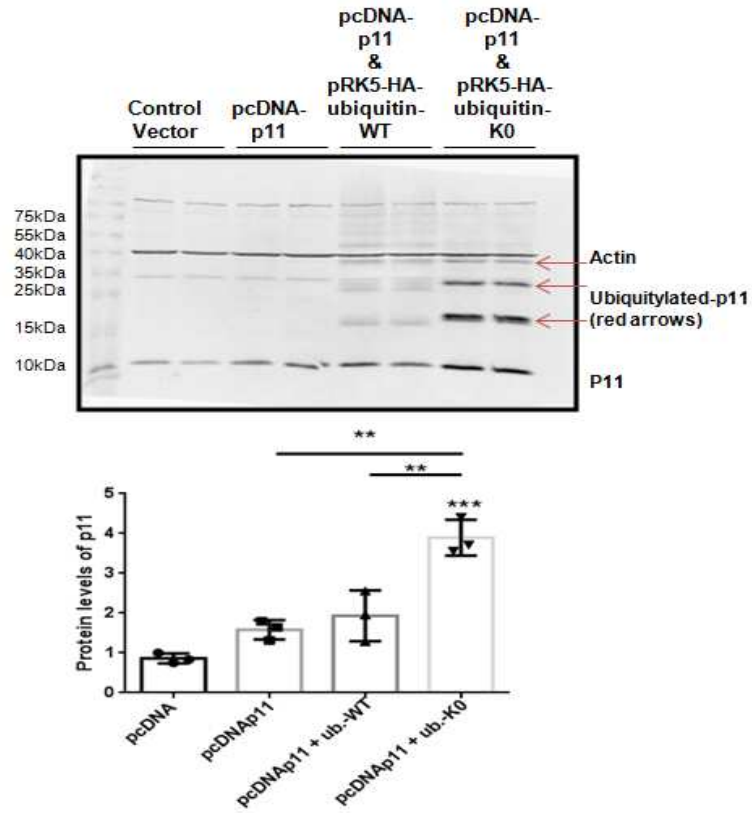


**Figure 26: Co-expression of p11 with ub-WT or ub-K0 in HEK293T cells upregulates p11 levels and produces higher molecular weight forms of p11.**

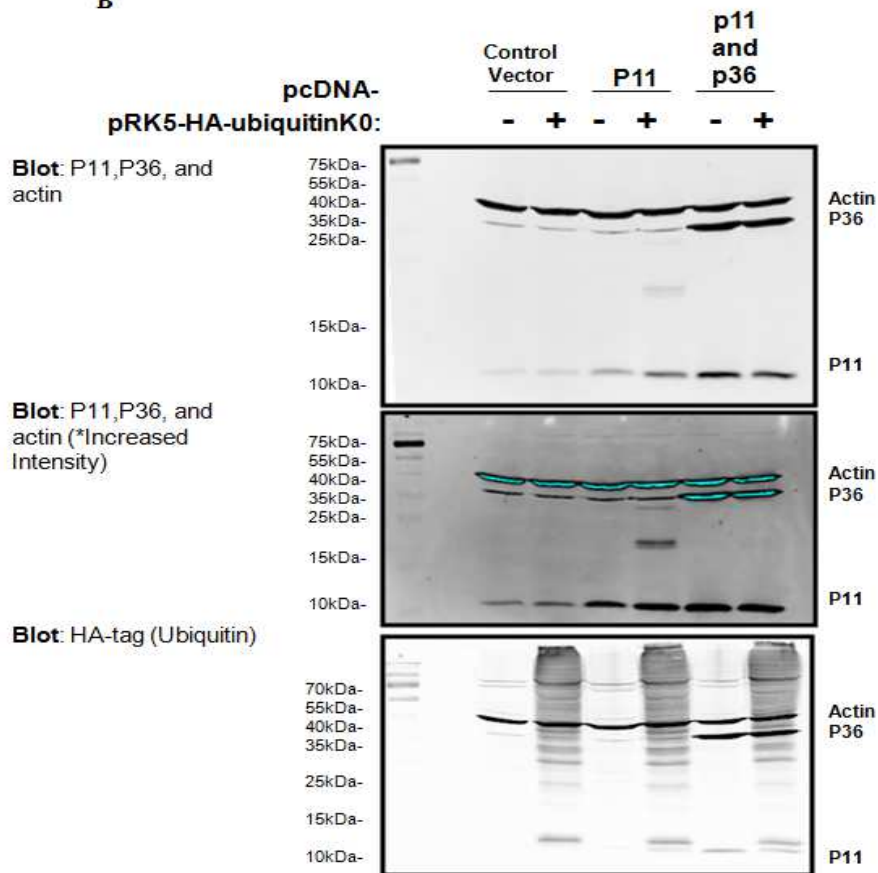
HEK293T cells were transiently transfected in 6-well plates using (A) 0.5 µg/well pcDNA3.1-empty control vector or pcDNA3.1-p11 vector alone or in combination with 2.5 µg/well pRK5-HA-ubiquitin wildtype (Ub-WT) or mutant, lysine-less ubiquitin (Ub-K0), and (B) 1.0 µg/well of pcDNA3.1-empty control vector, pcDNA-p11, or pcDNA and pcDNA-p36 vectors alone or in combination with 2.0 µg/well pRK5-HA-ubiquitin-K0. Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with β-actin used as a loading control. Quantification of protein levels by western blot analysis for (A) was performed as described in the methods section 2.11.2. Data is expressed as the mean ± S.D. of three independent experiments for (A). Statistical significance was determined for (A) using one-way ANOVA (with Tukey multiple comparisons), where \*\*P < 0.01 and \*\*\*P < 0.001 are considered statistically significance.



A



B



### **3.4.3. Lys57 identified as the site of ubiquitylation of p11**

Next, we examined if the higher molecular weight forms of p11 were ubiquitylated, and identified the sites of ubiquitylation on p11 using mass spectrometry analysis. Here, p11 was immunoprecipitated from HEK293T cells co-expressing p11 and ub-K0, p11 immunoprecipitates were resolved by SDS-PAGE, and the higher molecular weight forms of p11 were isolated from the acrylamide gel for mass spectrometry analysis. The mass spectrometry analysis indicated that the ~19.5 kDa band produced by the co-expression of p11 and ub-K0 (Figure 27D) was ubiquitylated on Lys57 (Figure 27A, C) and the ~28 kDa p11 band (Figure 27D) was ubiquitylated on Lys57 and several lysines including 27 or 37 (Figure 27B, C). The surface exposure of the lysine residues of p11 was examined to test the possibility that Lys57 was exposed; thereby indicating that ubiquitylation of p11 on this site was possible. Lys57 was 83.5% exposed (Figure 28), making it the most exposed lysine residue of p11, with the exception of the carboxy-terminal lysines from amino acids 93-97. This strongly indicated that ubiquitylation is likely to occur on Lys57 of p11.

**Figure 27: Lys57 identified as the site of ubiquitylation of p11.**

HEK293T cells were transiently transfected in 100cm<sup>2</sup> plates using 5 µg pcDNA3.1-p11 vector in combination with 25 µg/well of pRK5-HA-ub-K0. Cell lysates were prepared and p11 was immunoprecipitated from 2000 µg of protein. Immunoprecipitated proteins were resolved by SDS-PAGE and bands of the higher molecular weight species of p11 ((A) ~19 kDa and (B) ~28 kDa) were excised for ESI mass spectrometry analysis. Residues on the protein fragments that harbour a diglycine modification (1xGlyGly) identify the site of ubiquitylation. (C) Diagram highlighting the lysine residues identified that are ubiquitylated. (D) Diagram depicting that higher molecular weight species of p11 on a western blot and their corresponding ubiquitylation sites. Ryan Holloway prepared samples and Dr. Alex Cohen performed mass spectrometry.

A

Accession	Description	
P60903	Protein S100-A10 OS=Homo sapiens	
	Sequence	Modifications
	EFPGFLENQKDPLAVDK	
	IMKDLQCR	1xGlyGly [K3(100)]
	FAGDKGYLTK	
	DPLAVDK	
	EFPGFLENQK	

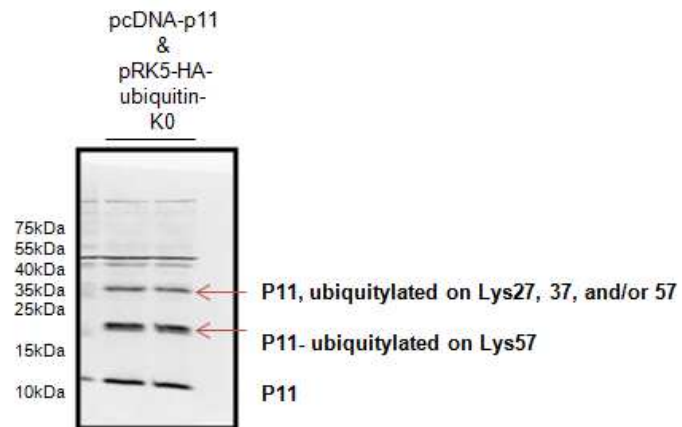
B

Accession	Description	
D3DV26	S100 calcium binding protein A10	
	Sequence	Modifications
	VLMEKEFPGFLENQK	1xGlyGly [K5]
	EFPGFLENQKDPLAVDK	
	IMKDLQCR	1xGlyGly [K3]
	GYLTKEDLR	1xGlyGly [K5]
	IMKDLQCR	1xGlyGly [K3]
	DPLAVDK	
	EFPGFLENQK	

C

**Ubiquitylation sites of p11:**  
 1 MPSQMEHAME  
 11 TMMFTFHKFA  
 21 GDKGYLT**K**ED  
 31 LRVLME**K**EFP  
 41 GFLENQKDPL  
 51 AVDKIM**K**DLD  
 61 QCRDGKVG**F**Q  
 71 SFFSLIAGLT  
 81 IACNDYFVVH  
 91 MKQKG**K**K

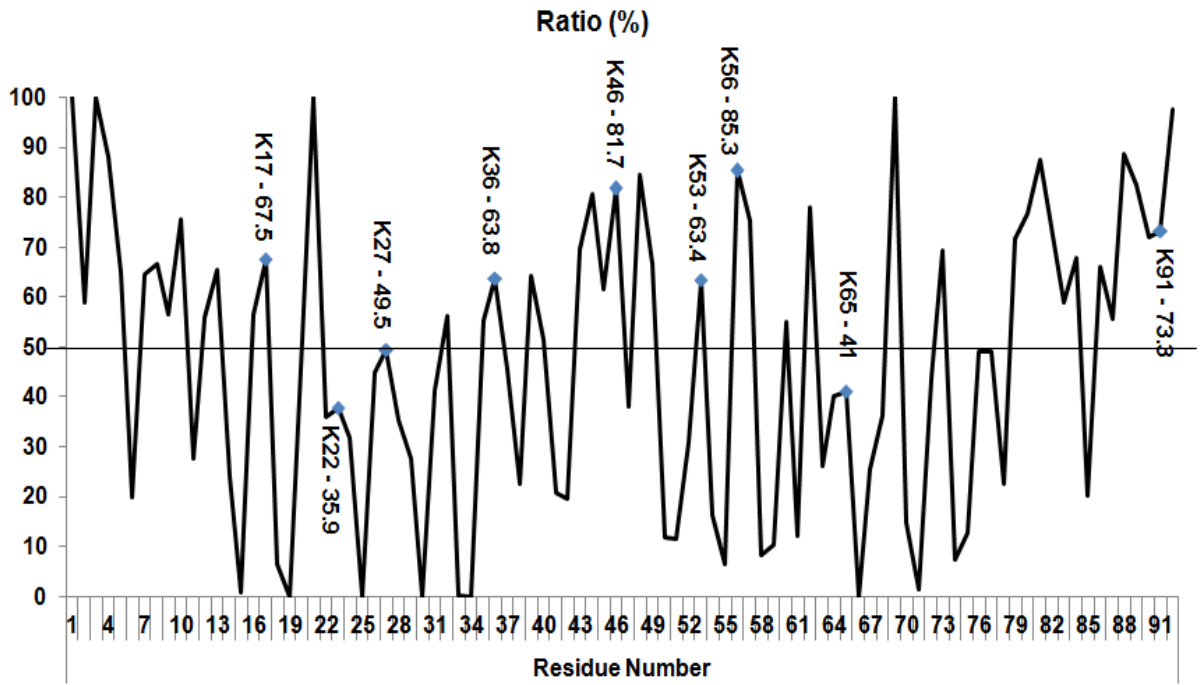
D



**Figure 28: Surface exposure of the lysine residues of p11 that were calculated using the online program, GetArea.**

(A) The GetArea online program was used to calculate the surface exposure of each amino acid of p11 (residues 1-92, with the first methionine residue removed) as a ratio of its exposed surface area in the crystal structure. Residues are considered solvent exposed if the ratio value exceeds 50%. (B) Diagram highlighting the surface exposed lysine residues of the p11 amino acid sequence, which was predicted using Get Area. The majority of the exposed lysine residues of p11 are contained within the central region (Lys 36-Lys56).

A



B

**Surface exposed lysine residues of p11 (in BOLD):**

1 MPSQMEHAME  
11 TMMFTFHKFA  
21 GDKGYLTKED  
31 LRVLMEKEFP  
41 GFLENQKDPL  
51 AVDKIMKDLD  
61 QCRDGKVGFG  
71 SFFSLIAGLT  
81 IACNDYFVVH  
91 MKQKGKK

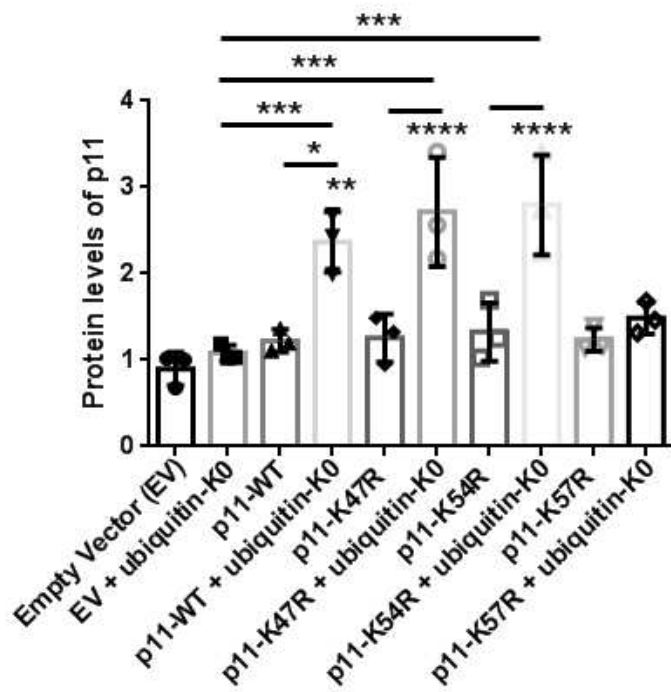
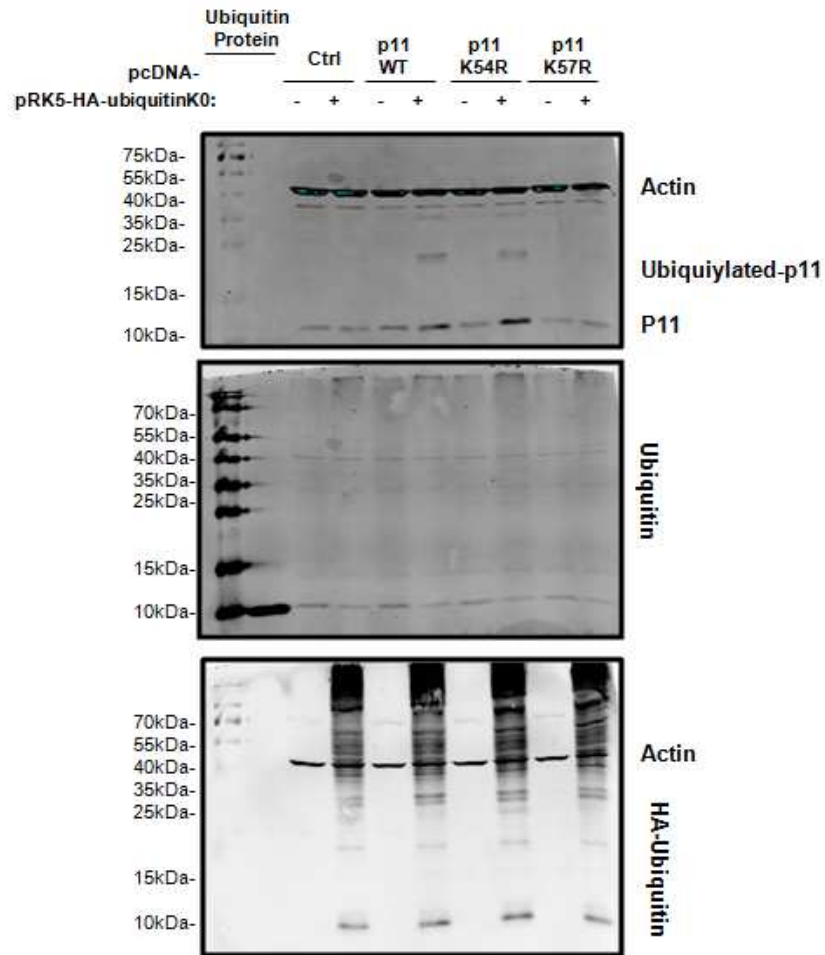
#### **3.4.4. Mutation of Lys57 (p11-K57R) prevents ubiquitylation of p11, but does not increase p11 protein levels**

Wagner *et al*<sup>284</sup> isolated p11 from various tissues and found ubiquitylated p11 on lysines 48, 54, and 57. Using mass spectrometry analysis, we determined that Lys57 is the primary site of ubiquitylation on p11. Mutants of p11 mutants were produced where the lysine was changed to arginine for lysine 54, and 57 (p11-K54R, and -K57R), which were the ubiquitylation sites of p11 determined by Wagner *et al*<sup>284</sup> and our mass spectrometry data. In order to validate the sites of ubiquitylation, ubiquitylated p11 was produced by co-transfecting HEK293T cells using vectors expressing ub-KO and p11-WT or the p11 mutants. We observed that forced expression of p11-WT or p11-K54R resulted in increased p11 expression when co-expressed with ub-KO (1.37±0.21-fold, P < 0.001 and 2.79±0.58-fold, P < 0.0001 respectively) and the presence of higher molecular weight forms of p11 were apparent. In contrast, co-expressing p11-K57R and ub-KO did not significantly increase p11 levels and higher molecular weight forms were not observed (Figure 29). These findings establish that forced expression of p11 and ubiquitin results in the ubiquitylation of Lys57 of p11.

**Figure 29: Ubiquitylation of p11 is prevented by the loss of Lys57.**

HEK293T cells were transiently transfected in 6-well plates using 0.5 µg/well pcDNA3.1-empty vector or p11-wildtype or p11 mutant (with Lys54 or Lys57 changed to arginine) vector alone or in combination with 2.5 µg/well of pRK5-HA-ub-K0. Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with β-actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean ± S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*P < 0.05, \*\*P < 0.01, and \*\*\* P < 0.001, \*\*\*\*P < 0.0001 are considered statistically significance.





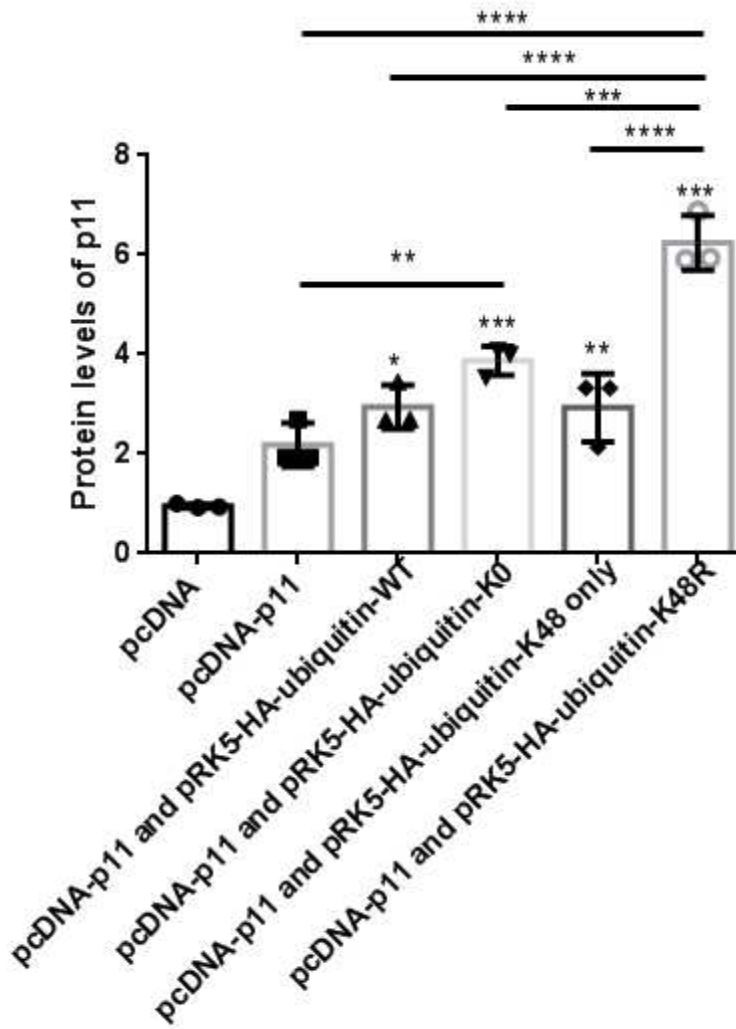
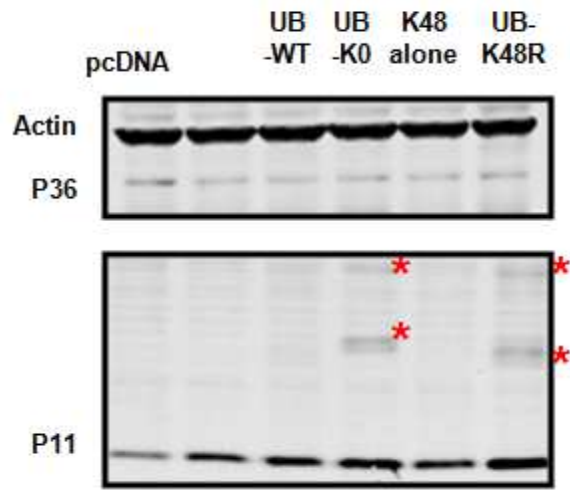
### **3.4.5. Loss of K48-ubiquitin linkages upregulates p11 expression**

We observed that co-expressing p11 and ub-K0 significantly increased p11 levels and produced multiple higher molecular weight forms of p11 even though the ub-K0 mutant cannot be polyubiquitylated. This indicates that multiple sites of ubiquitylation exist on p11. Since polyubiquitylation predominantly occurs by polyubiquitin linkages on Lys48 of ubiquitin, we examined whether the mutant ubiquitins with Lys48 changed to arginine (ub-K48R) or the loss of all lysines except Lys48 (ub-K48 only) would also significantly increase p11 levels and produce multiple higher molecular weight forms of p11. The forced expression of p11-WT increased p11 expression when co-expressed with ub-K0 ( $3.87 \pm 0.29$ -fold,  $P < 0.001$ ) and ub-K48R ( $6.24 \pm 0.55$ -fold,  $P < 0.0001$ ) compared to the control group, the presence of higher molecular weight forms of p11 were apparent (Figure 29). The co-expression of p11 with ub-K0 or ub-K48R significantly upregulated p11 expression by 1.77-fold and 2.86-fold respectively compared to the cells co-expressing p11 with ub-WT. No difference in the expression of p11 was found between cells co-expressing p11 with ub-WT or ub-K48 alone, and higher molecular weight forms were not observed (Figure 30). This suggests the Lys48-polyubiquitin linkages regulate p11 protein levels, which may occur indirectly by transcriptional or post-translational effects (discussed further in Chapter 5: Discussion).

**Figure 30: Co-expression of ubiquitin mutants lacking Lys48 and p11 in HEK283T cells results in the increase p11 expression.**

HEK293T cells were transiently transfected in 6-well plates using 0.5 µg/well pcDNA3.1-empty vector or p11-wildtype vector alone or in combination with 2.5 µg/well of pRK5-HA-ub-WT, -K0, -K48 only, or -K48R vectors. Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with β-actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean ± S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001, \*\*\*\*P < 0.0001 are considered statistically significance.

pcDNA-p11 and



### **3.5. The p36 protein regulates p11 protein levels but not p11 transcript levels.**

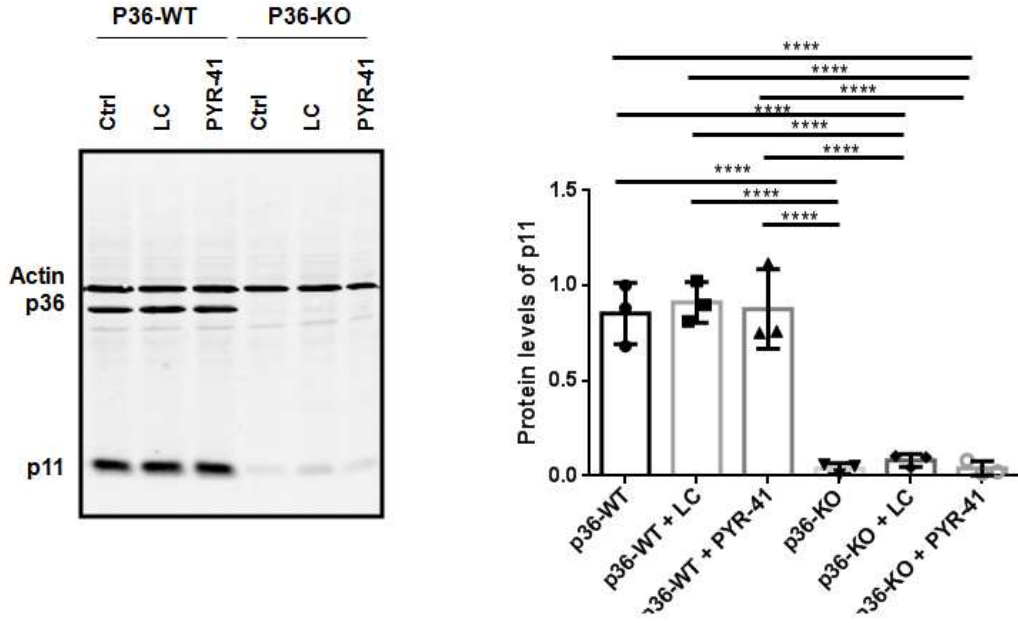
#### **3.5.1. Inhibition of proteasomal degradation or ubiquitylation does not restore p11 protein levels in macrophages isolated from p36<sup>-/-</sup> mice or HEK293T cells.**

Understanding the mechanisms regulating p11 expression in APL and its treatment by ATRA is complicated by the fact that p11 is only stable after forming a complex with p36, thus implying that agents that affect p36 protein levels will also affect p11 protein levels. In the absence of p36, p11 has been reported to be unstable and is rapidly ubiquitinated and degraded by the 26S proteasome<sup>83,90,91</sup>. The simplest experimental paradigm for examining the mechanism by which p36 protects p11 is to examine p11 mRNA and protein levels in cells isolated from p36 knockout mice. Hence, cell cultures of macrophages isolated from p36-wildtype (p36<sup>+/+</sup>) or p36-knockout (p36<sup>-/-</sup>) mice were incubated in the presence or absence of LC for 24 h. As shown in figure 31A, total depletion of p36 resulted in a dramatic loss of p11 protein. Surprisingly, p11 protein expression was not rescued by LC treatment, suggesting that the loss of p11 was not due to proteasomal degradation. Next, we repeated this analysis with HEK293T cells that express low levels of p11 and p36. The incubation of HEK293T cells with LC failed to affect the levels of p11 protein levels (Figure 31B), indicating that the proteasome did not regulate p11 levels in these cells.

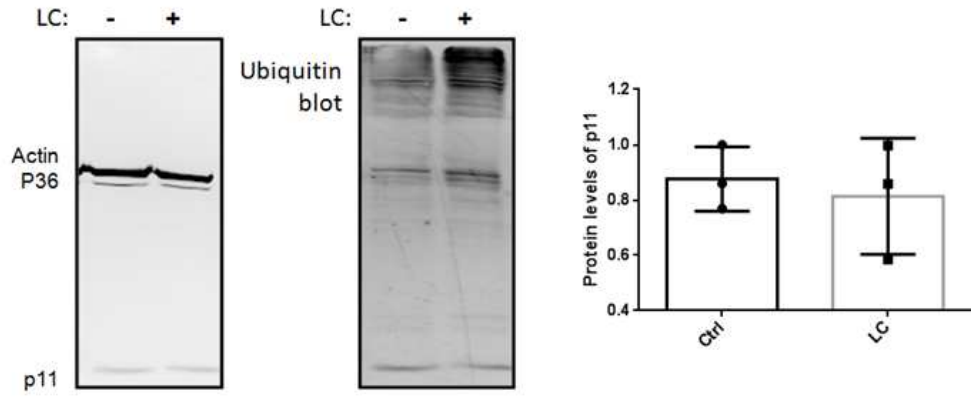
**Figure 31: Inhibition of proteasomal degradation or ubiquitylation has no effect on p11 protein levels in cells with low levels of p36 protein or an absence of p36 protein.**

We performed western blot analysis of (A) peritoneal macrophages isolated from p36<sup>+/+</sup> and p36<sup>-/-</sup> mice treated for 24 h using 3  $\mu$ M LC or 10  $\mu$ M PYR-41, and (B) HEK293T cells treated for 16 h using 2.5 $\mu$ M lactacystin (LC). Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*\*\*\*P < 0.0001 is considered statistically significance.

A



B



### **3.5.2. Overexpression of p36 upregulates p11 protein but not transcript levels**

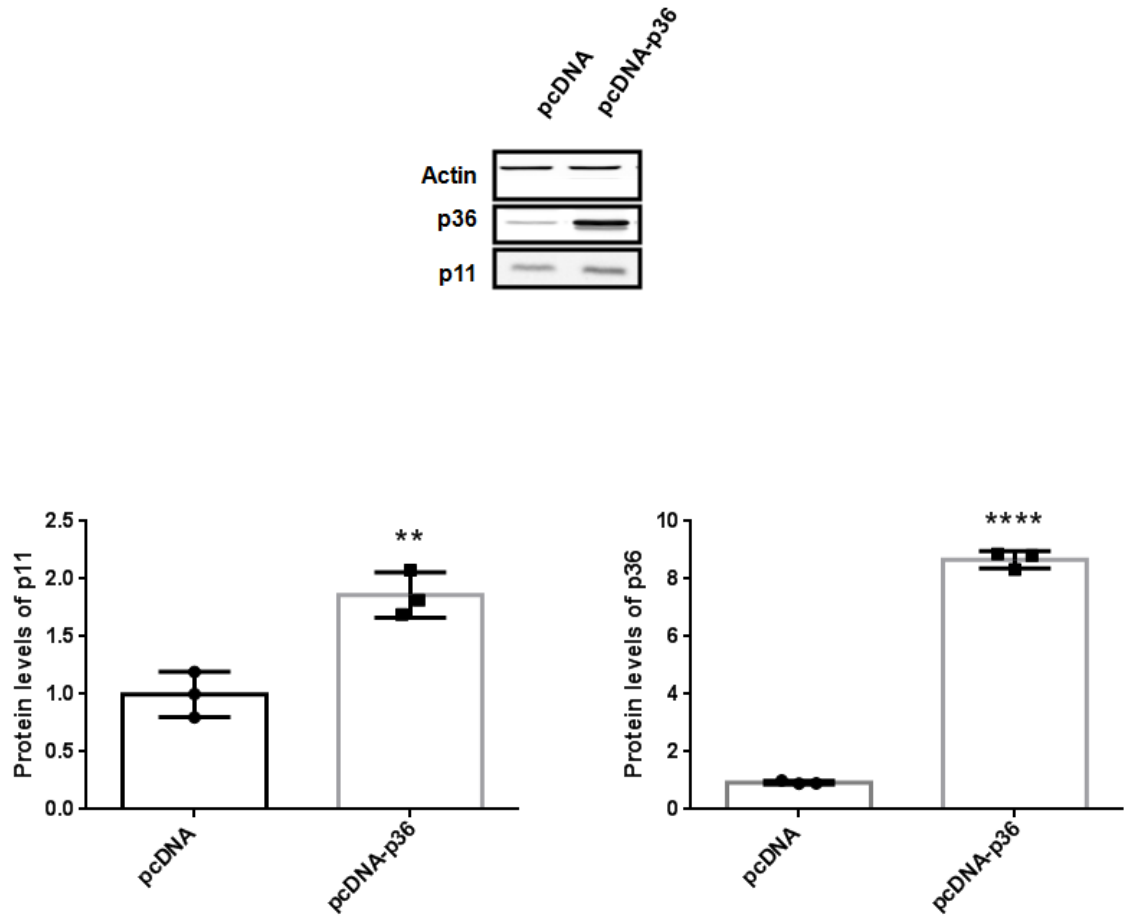
Proteasomal inhibition did not prevent the loss of p11 in the absence of p36 or in HEK293T cells that have low expression of p11 and p36; however, we did observe that co-expression of p11 and p36 increased p11 protein expression and prevented p11 ubiquitylation. We reasoned that the forced expression of p36 in the presence of low intracellular levels of p11 and p36 would result in the accumulation of p11 protein since p36 was available to protect p11 from proteasomal degradation. Furthermore, we examined whether the increased p11 protein levels may also be attributed to the upregulation of p11 mRNA levels by p36 overexpression. The overexpression of p36 resulted in a  $1.86 \pm 0.2$ -fold ( $P < 0.001$ ) increase of p11 protein levels (Figure 32A), but did not affect p11 mRNA levels (Figure 32B). Because the incubation of HEK293T cells with LC failed to affect the levels of p11 protein levels, this indicated that the proteasome did not regulate p11 levels in these cells and that the increased protein levels of p11 observed in the response of the forced expression of p36 was not due to p36-dependent protection of p11 from proteasomal degradation.



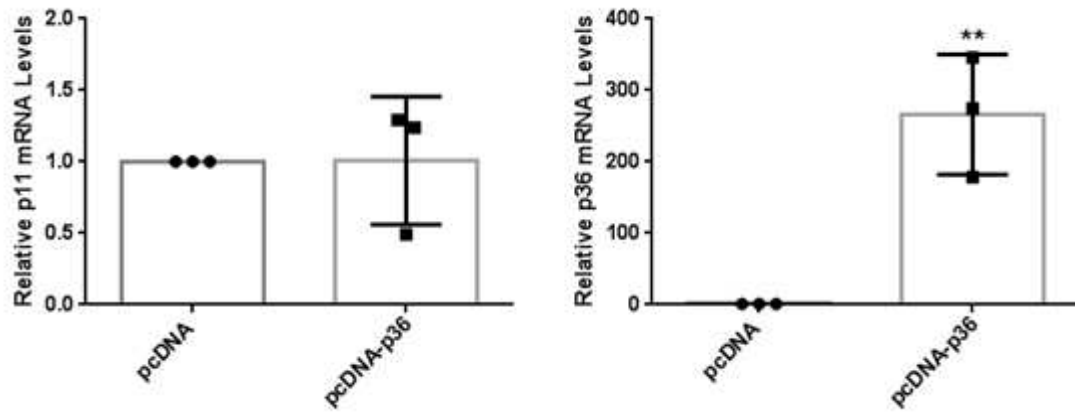
**Figure 32: Overexpression of p36 in HEK293T cells upregulates p11 mRNA and protein levels.**

(A) HEK293T cells were transiently transfected in 6-well plates using 2.0 µg/well pcDNA3.1-empty vector or pcDNA3.1-p36 vector. Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with β-actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Total RNA extracted from (B) HEK293T cells were transiently transfected in 6-well plates using 2.0 µg/well pcDNA3.1-empty vector or pcDNA3.1-p36 vector were used for cDNA synthesis. The relative expression of p11 and p36 mRNA levels was determined from cDNA (25 ng) by qPCR analysis and normalized to GAPDH, β-actin and HPRT1. Data is expressed as the mean ± S.D. of three independent experiments. Statistical significance was determined using the Student t-test for unpaired observations, where \*\*P < 0.01 and \*\*\*\*P < 0.0001 are considered statistically significant.

A



B



### **3.6. ATRA induces the loss of p11 independent of the presence of the PML/RAR $\alpha$ oncoprotein in the MCF-7 breast cancer cell line**

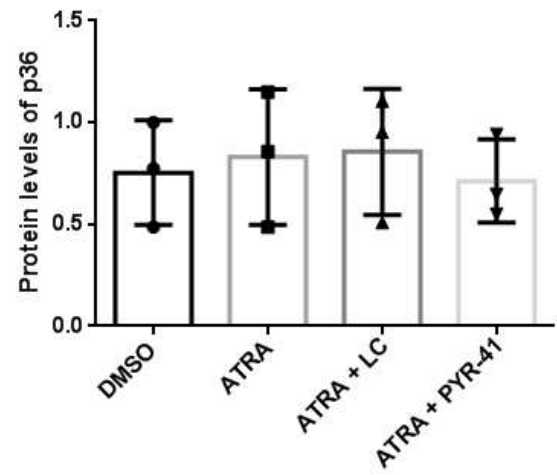
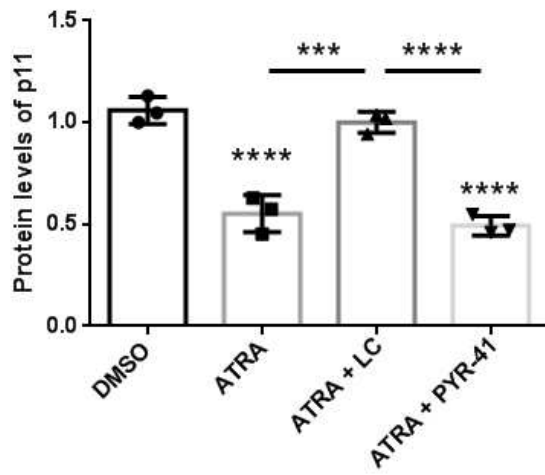
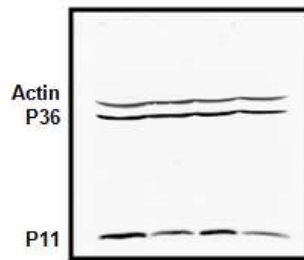
#### **3.6.1. ATRA induces ubiquitin-independent proteasomal degradation of p11 in MCF-7 cells**

To examine if ATRA could directly regulate p11 independent of PML/RAR $\alpha$ , we chose the MCF-7 cell line since these cells do not express this oncoprotein. MCF-7 cells were treated with ATRA alone or in combination with LC or PYR-41. Although ATRA treatment of MCF-7 cells had no effect on p36 protein levels, p11 was significantly decreased by  $1.82 \pm 0.09$ -fold ( $P < 0.01$ ). In addition, the presence of LC, but not PYR-41, prevented the ATRA-induced loss of p11 expression in MCF-7 cells (Figure 33). In MCF-7 treated with ATRA and LC, we also did not observe the presence of higher molecular weight species of p11 that would indicate it is ubiquitylated. Consistent with this observation, findings obtained by Johansson and colleagues suggested that retinoic acid signalling regulated the expression of p11 and several other members of the S100 family according to proteomic analysis of MCF-7 breast cancer cells<sup>285</sup>.

**Figure 33: ATRA downregulated p11 expression in MCF-7 breast cancer cells by ubiquitin-independent proteasomal degradation.**

Western blot analysis of MCF-7 cells treated for 48 h with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M LC or 2  $\mu$ M PYR-41. Cell lysates were prepared and the level of the indicated proteins was examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*\*\*P < 0.001 and \*\*\*\*P < 0.0001 are considered statistically significant.

PYR-41:	-	-	-	+
LC:	-	-	+	-
ATRA:	-	+	+	+

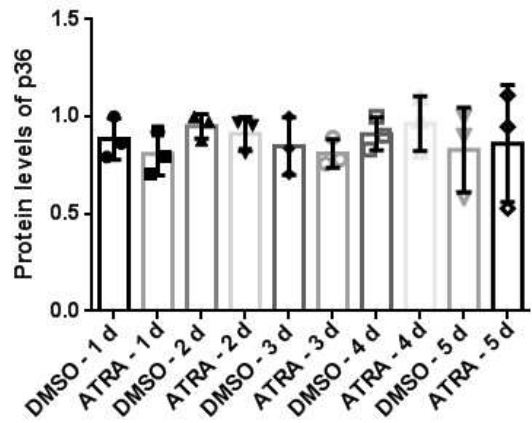
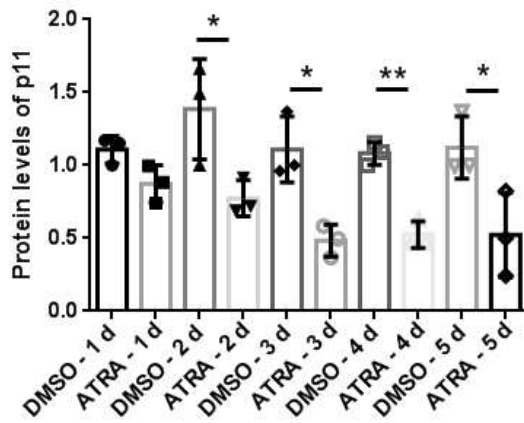
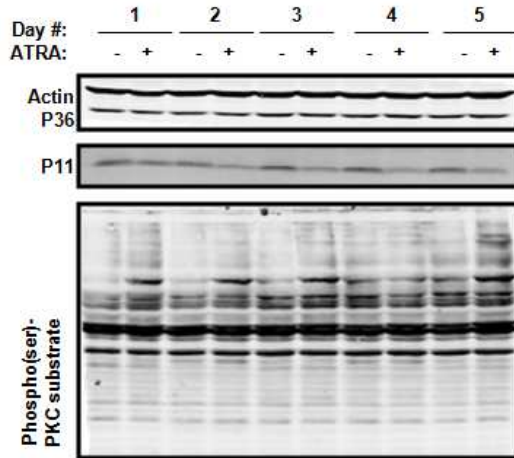


### **3.6.2. ATRA downregulation of p11 protein levels correlates with increased PKC activation in MCF-7 cells**

Because ATRA induced the loss of p11 without affecting p36 in MCF-7 cells, this suggested that the p11-p36 interaction may be disrupted by PKC phosphorylation and that this would result in the decrease of p11 due to proteasomal degradation. Hence, ATRA-induced PKC activity was also assessed using MCF-7 cells. The cells were treated with and without ATRA each day over the course of 5 days. Western blot analysis indicated that p36 protein levels were not affected by ATRA treatment; however, p11 expression significantly decreased by  $1.82 \pm 0.12$ -fold ( $P < 0.05$ ) after two days of treatment and decreased by  $2.13 \pm 0.29$ -fold ( $P < 0.05$ ) on day 5. In addition, ATRA treatment increased phosphoserine PKC substrate levels on each day of treatment (Figure 34). Similar to our observations in ATRA-treated NB4 cells, this result demonstrates that ATRA upregulates PKC activity concomitant with the loss of p11 protein level.

**Figure 34: ATRA induces PKC activity concomitant with the decrease of p11 expression in MCF-7 cells.**

MCF-7 cells were treated with 1  $\mu$ M ATRA or a vehicle control for the indicated times. Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using the Student t-test for unpaired observations, where \*P < 0.05 and \*\*P < 0.01 are considered statistically significant. The Student t-test was performed for each day in which p11 or p36 expression of ATRA-treated NB4 cells was compared to the control group.





### **3.6.3. Inhibition of PKC activity does not prevent the ATRA-induced loss of p11 in MCF-7 cells**

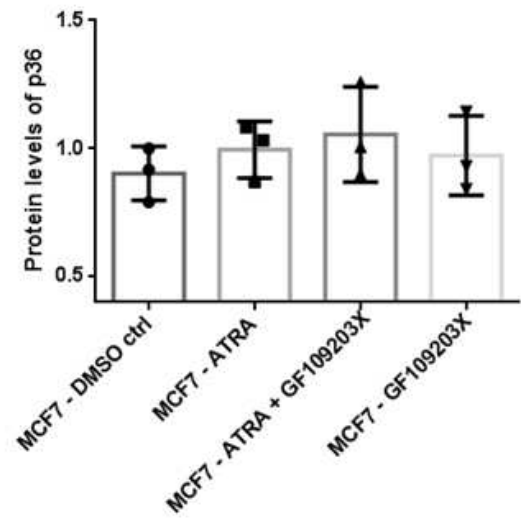
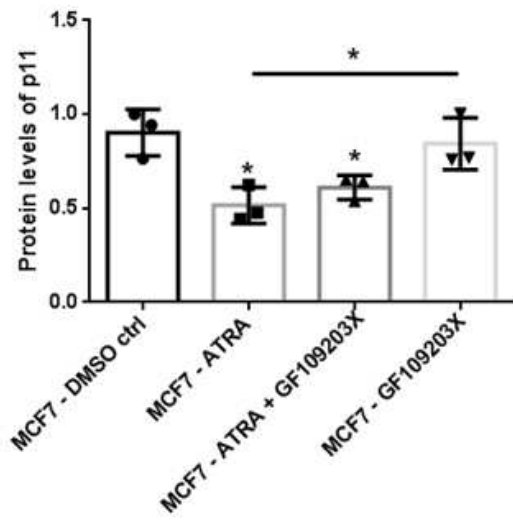
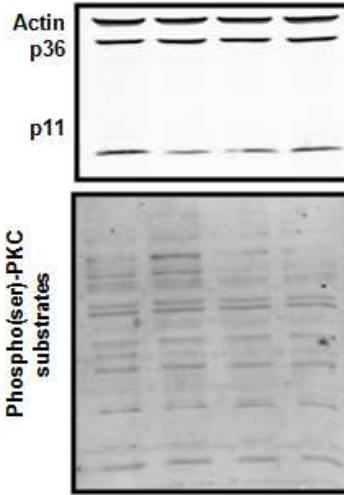
We observed a correlation between increased PKC activity and the loss of p11 in NB4 and MCF-7 cells. Inhibition of PKC activity did not prevent the ATRA-induced loss of p11 in NB4 cells, which may be attributed to the loss of p36. In MCF-7 cells, ATRA did not affect p36 protein levels, yet p11 protein levels were decreased. This suggested that the ability of p36 to prevent the proteasomal degradation of p11 was inhibited. Since PKC phosphorylation of p36 on serines 11 and 25 was proposed to prevent the p11-p36 interaction and promote the proteasomal degradation of p11, we examined if inhibition of PKC activity could prevent the ATRA-induced loss of p11 in MCF-7 cells. The involvement of PKC activity in the ATRA-induced loss of p11 protein levels was examined using MCF-7 cells treated with ATRA alone or with GF109203X. Similar to the result observed in NB4 cells, the presence of GF109203X did not prevent the downregulation of p11 in ATRA-treated MCF-7 cells (Figure 35), indicating that the conventional PKC isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) are not involved in the ATRA-induced loss of p11 expression in MCF-7.

**Figure 35: PKC inhibition does not prevent the ATRA-induced loss of p11 in MCF-7 cells.**

MCF-7 cells were treated with 1  $\mu$ M ATRA alone or in combination with 1 $\mu$ M of GF109203X for 48 h. Cell lysates were prepared and the levels of the indicated proteins were examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*P < 0.05 is considered statistically significant.

MCF-7 cells

ATRA:	-	+	+	-
GF109203X:	-	-	+	+

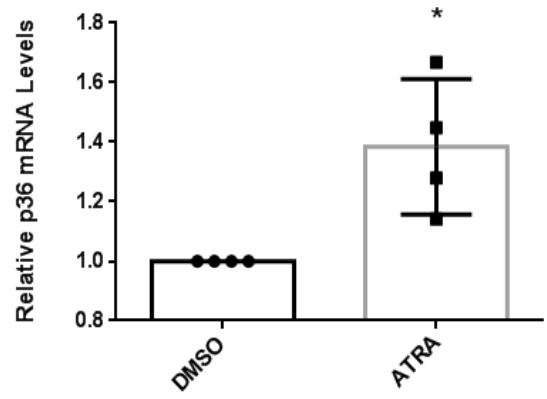
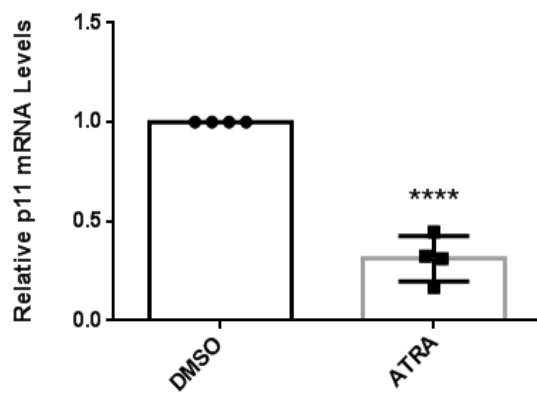


#### **3.6.4. ATRA treatment downregulates p11 mRNA levels in MCF-7 breast cancer cells**

We observed that ATRA reduced p11 protein and mRNA levels in NB4 cells, and we predicted that this is likely due to the ATRA-induced loss of PML/RAR $\alpha$ . However, we also observed that ATRA reduced p11 proteins levels, but not p36 protein levels, in MCF-7 cells, indicating that ATRA treatment affected p11 expression independently of its effect on PML/RAR $\alpha$  and/or p36. Hence, we next examined ATRA induced the downregulation of p11 mRNA levels in addition to the downregulation of p11 protein levels in MCF-7 cells. P11 mRNA levels were reduced by 3.21  $\pm$ 0.11-fold ( $P < 0.0001$ ) and p36 was increased by 1.38 $\pm$ 0.23-fold ( $P < 0.01$ ) in ATRA-treated MCF-7 cells compared to the control group (Figure 36). Here, ATRA downregulated p11 mRNA levels in the absence of PML/RAR $\alpha$ , yet MCF-7 cells express wildtype form of RAR $\alpha$ , the receptor of ATRA. This result suggests that ATRA regulates p11 transcription possibly through RAR $\alpha$  activation.

**Figure 36: ATRA downregulates p11 mRNA levels in MCF-7 cells.**

Total RNA extracted from ATRA-treated (48 h) MCF-7 cells was used for cDNA synthesis. The relative expression of p11 and p36 mRNA levels was determined from cDNA (25 ng) by qPCR analysis and normalized to GAPDH,  $\beta$ -actin and HPRT1. Data is expressed as the mean  $\pm$  S.D. of four independent experiments. Statistical significance was determined using the Student t-test for unpaired observations, where \*P < 0.05 and \*\*\*\*P < 0.0001 are considered statistically significance.



### **3.6.5 Retinoic Acid Receptor Element (RARE) predicted near the transcriptional start site of p11**

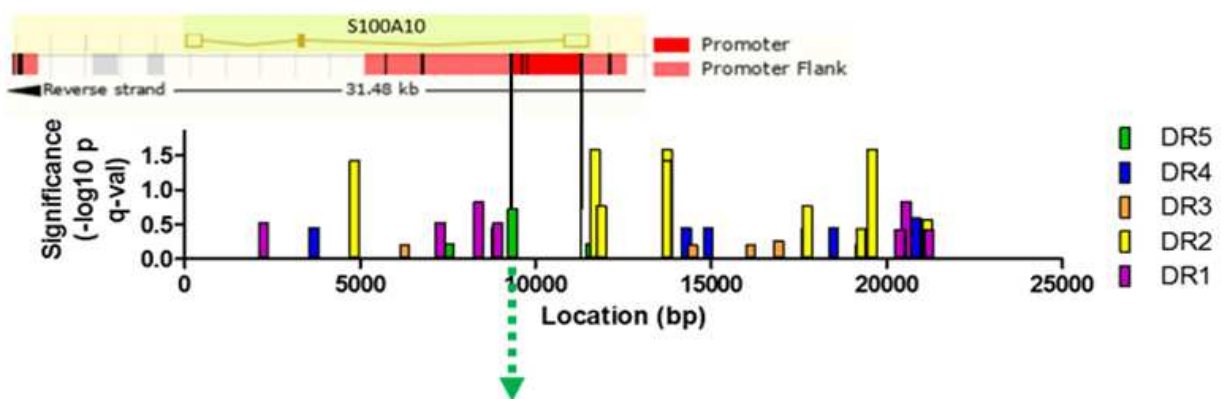
ATRA downregulated p11 mRNA levels independent of its effect on PML/RAR $\alpha$ , suggesting that p11 may be regulated by the ATRA receptor RAR $\alpha$ , which mediates the transcriptional activities by the retinoic acid signalling pathway. Hence, the p11 promoter was examined to identify if there are any potential RAR $\alpha$  binding motifs. *In silico* analysis of the  $\pm 10$  kb region from the p11 transcriptional start site showed several potential binding sites for RAR $\alpha$  and RAR $\gamma$  as characterized by canonical retinoic acid response element (RARE) hexameric [RGKTSA] repeats separated by 5-bp direct repeats (DR5) (Figure 37, Table 7). This indicates that p11 transcription can be directly regulated by retinoic acid transcription factors.

**Figure 37: RAR-bind motifs in the p10±kB region of the p11 promoter.**

P11 coding sequence and promoter as identified in ENSEMBL. The sequence ±10 kb of the p11 transcription start site contains several direct repeat (DR) elements with 1-5 nucleotides separating the canonical RARE hexameric [RGKTSA] repeats; identified via FIMO (Find Individual Motif Occurrences; MEME-Suite). Height of the bar representing each DR element is indicative of how well the DR element in the sequence matches the DR sequence which was anticipated (i.e. highly significant when the sequence was a perfect match to the predicted DR element). Within the promoter region of p11, a DR5 RARE was found and (B) Tomtom (MEME-suite) analysis predicts a significant association with either/both RARG & RARA. The most likely RARG motif is illustrated in the sequence logo; the top logo is the RARG\_full\_2 sequence from the Jolma2013 database with the lower sequence representing the sequence found in the p11 promoter region. The repeating hexameric [RGKTSA] motif is shown within the black boxes separated by 5 nucleotides; the height of each residue is indicative of their relative frequency within the sequence. Margaret Thomas performed analysis of the p11 promoter.



A) Promoter region of S100A10



B) DR5 element likely associates with RARG and/or RARA

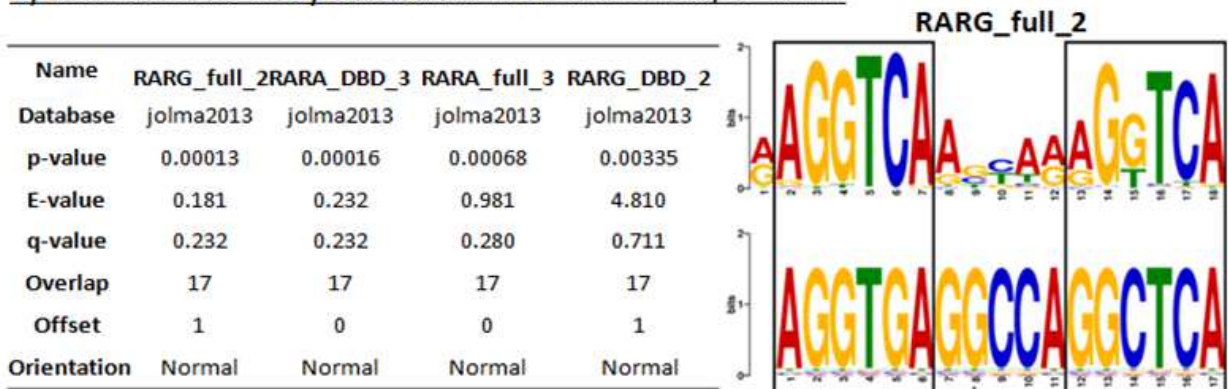


Table 7: Table of RAR $\alpha$  binding motifs, direct repeats (DR) 1-5.

MotifID	Motif Sequence	Start	Stop	Strand	Score	p-value	q-value	Matched Sequence		
DR5	RGKTSAnnnnnRGKTS	7863	7879	-	13.2121	4.78E-06	0.19	AGGTGA	GGCCA	GGCTCA
		19345	19361	+	11.7212	5.49E-05	0.603	ATGTGA	GAATG	GGGTGA
		17872	17888	+	11.2303	7.88E-05	0.603	GGTTGA	CTTTT	AAGTGA
		17783	17799	+	11.1939	8.11E-05	0.603	AGGTGA	AAATA	GGTTGT
		6045	6061	-	10.6606	9.06E-05	0.603	ACTTCA	ATAAA	AGGTGA
		10102	10118	+	10.6303	9.09E-05	0.603	ACTTGA	TCAAT	AGTTCA
DR4	RGKTSAnnnnRGKTS	10206	10221	+	13.1091	5.12E-06	0.167	GGATCA	CCTC	AGGTCA
		18109	18124	+	12.8424	8.44E-06	0.167	AGATCA	CCTG	AGGTCA
		19356	19371	+	12.4606	1.92E-05	0.254	GGGTGA	AGCT	GGGAGA
		12808	12823	-	11.8727	4.53E-05	0.352	AGGCCA	AGAT	GGGTGA
		6910	6925	-	11.6242	5.86E-05	0.352	AGTTGG	TGAG	AGGTGA
		2214	2229	-	11.5515	6.90E-05	0.352	GGGTGA	TTGC	ATTTGA
		16247	16262	+	11.3697	7.22E-05	0.352	AGTAGA	TTTT	GGGTCA
		13448	13463	-	11.3515	7.46E-05	0.352	AGTACA	GTAC	AGTTGA
		17005	17020	-	11.297	8.00E-05	0.352	ACTTCA	CTGA	AGGTGA
DR3	RGKTSAnnnRGKTS	7399	7414	-	10.7697	9.03E-05	0.358	AGTTCA	AAAA	ATGTGA
		15447	15461	-	12.6545	1.38E-05	0.548	GGTTCA	TAG	TGGTGA
		4793	4807	+	12.0424	3.36E-05	0.63	AGGAGA	GCA	AGGTGA
		12998	13012	-	11.703	5.75E-05	0.63	AGTTGC	TCT	GGGTGA
DR2	RGKTSAnnRGKTS	14654	14668	+	11.6667	6.33E-05	0.63	AGTTCA	CAC	AGGTCT
		12270	12283	+	17.0788	1.62E-06	0.0257	AGGTCA	AC	AGGTCA
		10216	10229	+	17.0667	1.94E-06	0.0257	AGGTCA	GG	AGTTCA
		18119	18132	+	17.0667	1.94E-06	0.0257	AGGTCA	GG	AGTTCA
		3368	3381	-	13.1091	4.70E-06	0.0374	GGATCA	CG	AGGTCA
		12262	12275	+	13.1091	4.70E-06	0.0374	GGATCA	CG	AGGTCA
		10395	10408	+	12.1697	2.86E-05	0.169	GGGTGA	CA	GAGTGA
		16257	16270	+	12.1576	2.98E-05	0.169	GGGTCA	TT	GGGGCA
		19689	19702	-	11.8121	5.35E-05	0.266	AGGTCA	CC	ACGTCA
DR1	RGKTSAnRGKTS	17794	17807	+	11.2788	8.33E-05	0.368	GGTTGT	TA	AGTTGA
		19074	19086	+	13.2606	3.71E-06	0.146	GGGTGA	G	GGATGA
		6910	6922	-	12.9152	7.39E-06	0.146	TGGTGA	G	AGGTGA
		779	791	-	12.303	2.46E-05	0.296	GGGTGA	G	AGGAGA
		5812	5824	-	12.103	3.12E-05	0.296	CGTTCA	T	AGTTGA
		7449	7461	-	12.0061	3.74E-05	0.296	AGGTCA	G	GGTGGA
		18925	18937	+	11.6667	6.50E-05	0.375	GGGTCA	G	ATTTGA
		19735	19747	+	11.6545	6.64E-05	0.375	AGTTGT	C	GGTTCA
18883	18895	+	11.4182	7.64E-05	0.378	ACTTCA	T	GGGTGA		

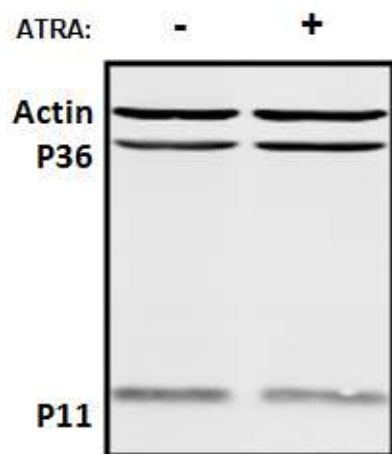
### **3.6.6. Expression of p11 is not affected by ATRA treatment of triple-negative breast cancer cell lines, MDA-MB-231 and SUM159PT**

The effect of ATRA on p11 expression was also assessed in triple negative breast cancer cell lines (MDA-MB-231 and SUM159PT); however, p11 protein levels were not affected (Figure 38). Thus, ATRA treatment can promote p11 proteasomal degradation independent of PML/RAR $\alpha$  and p36, although this phenomenon is cell context dependent.

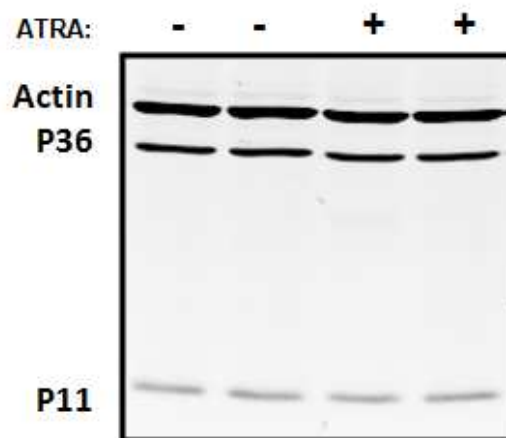
**Figure 38: ATRA does not affect p11 or p36 expression in triple negative breast cancer cell lines, MDA-MB-231 and SUM159PT.**

(A) MDA-MB-231 and (B) SUM195PT cells were treated with 1  $\mu$ M ATRA or a vehicle control for 48 h. Cell lysates were prepared and expression of the indicated proteins were examined by western blot analysis with  $\beta$ -actin was used as a loading control. Data is expressed as three independent experiments.

**A** MDA-MB-231



**B** SUM159PT

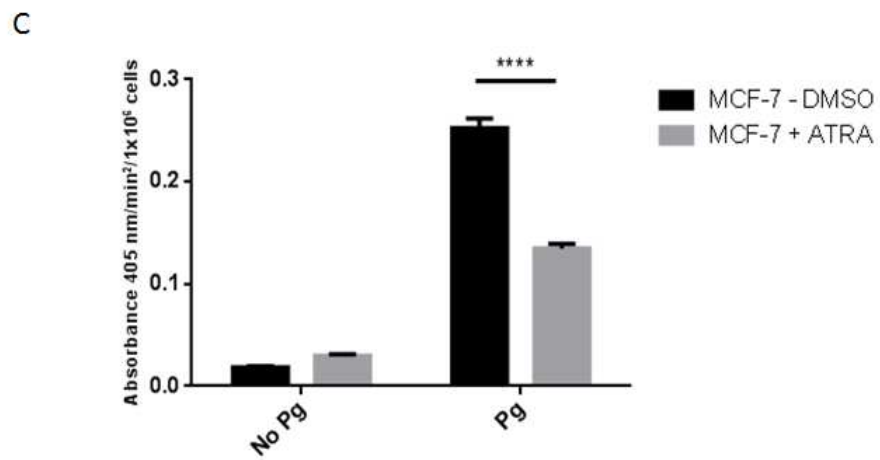
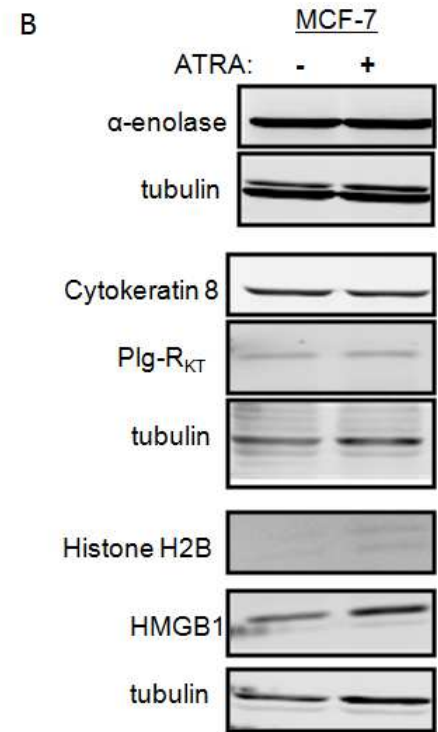
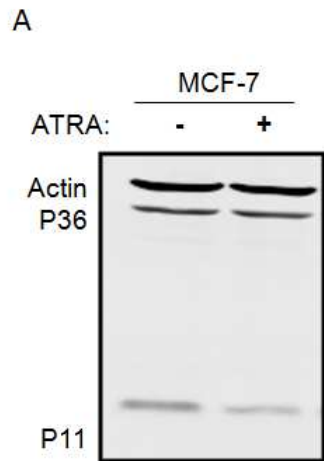


### **3.6.7. ATRA treatment reduces rate of plasmin generation in MCF-7 cells**

Since ATRA downregulates p11 in MCF-7 cells, we expected that the loss of p11 would result in the downregulation of plasmin generation. First, the effect of ATRA on the expression of p11, p36 and several other plasminogen receptors was assessed in MCF-7 cells to determine that any downregulation of plasmin generation occurring in ATRA-treated MCF-7 cells was due to the reduction of p11 and no other plasminogen receptors. Although ATRA treatment downregulated p11 expression as expected (Figure 39A), it did not affect the expression of other plasminogen receptors (Figure 39B) or p36. Because the only plasminogen receptor affected by ATRA was p11, we would expect that ATRA-induced downregulation of p11 in MCF-7 cells would reduce the ability of the cells to produce plasmin. As expected, the rate of plasmin generation was downregulated by  $1.96 \pm 0.005$ -fold ( $P < 0.0001$ ) in ATRA-treated MCF-7 cells compared to the DMSO vehicle treated control cells (Figure 39C), demonstrating that ATRA can reduce p11 and plasmin generation in cancers other than APL.

**Figure 39: ATRA treatment downregulates p11 and plasmin generation in MCF-7 cells without affecting the expression of other plasminogen receptors.**

Cell lysates were prepared from MCF-7 cells treated with 1  $\mu$ M ATRA for 72 h. The level of (A) p11 and p36 and (B) other plasminogen receptors (histone H2B, HMGB1, Plg-R<sub>KT</sub>,  $\alpha$ -enolase, and S100A4) was examined by western blot analysis with  $\beta$ -actin or  $\beta$ -tubulin used as loading controls. Data is expressed as three independent experiments. (C) MCF-7 cells treated with ATRA for 72 h were washed 3 times with Hanks balanced salt solution (HBSS containing 3 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>). The cells were resuspended in HBSS without or with 0.5  $\mu$ M glu-plasminogen. Cells were seeded to a 96-well plates (100, 000 cells per well) and incubated for 20–30 minutes before the addition of 500  $\mu$ M plasmin chromogenic substrate, S2251. The final volume was 200  $\mu$ L per well. Cleavage of S2251 was measured as absorbance at 405 nm for every 4 min over 4 h. The rate of plasmin generation was measured from the A405 nm vs. min<sup>2</sup> progress curves (n = 3). Data is expressed as 405 nm/min<sup>2</sup>/1 x 10<sup>6</sup> cells. Error bars represent the standard error of the mean (S.E.M.) obtained from three independent experiments with at least triplicate samples each. Statistical significance was determined using the Student t-test for unpaired observations, where \*\*\*\*P < 0.0001 is considered statistically significant. Western blot analysis of p11 and p36 was performed by Ryan Holloway, western blots for other plasminogen receptors and Dr. Alamelu (Dharini) Bharadwaj performed the plasmin generation assay.





**CHAPTER 4: RESULTS - INVOLVEMENT OF P11 IN FIBRINOLYSIS AND  
COAGULATION IN APL**

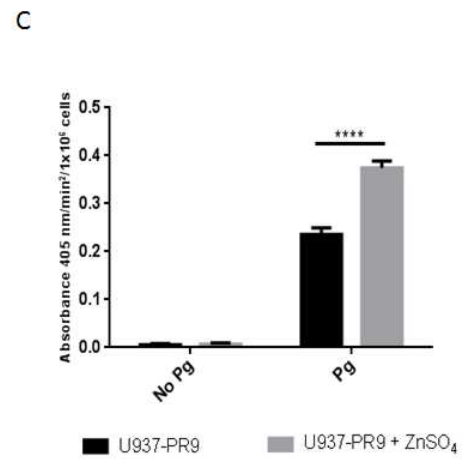
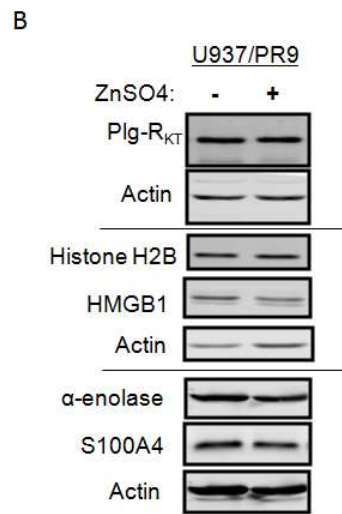
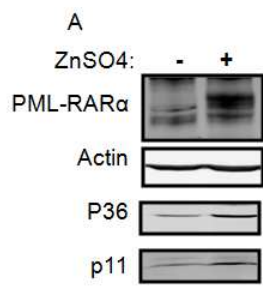
**4.1. Regulation of plasmin activity by the PML/RAR $\alpha$  oncoprotein and ATRA treatment**

**4.1.1. Induction of PML/RAR $\alpha$  increases the rate of plasmin generation**

PML/RAR $\alpha$  has been shown to promote fibrinolytic activity through the upregulation of plasmin activity<sup>146</sup> and p11 and p36 expression<sup>125,286</sup> in APL promyelocytes and PR9 cells with induced expression of PML/RAR $\alpha$ . To elucidate the role of PML/RAR $\alpha$  in regulating the expression of p11 and other plasminogen receptors, the expression of p11, p36 and other plasminogen receptors was assessed in PML/RAR $\alpha$ -induced PR9 cells. The induction of PML/RAR $\alpha$  upregulated p11 and p36 expression (Figure 40A), but the expression of other plasminogen receptors was not affected (Figure 40B). Furthermore, the induction of PML/RAR $\alpha$  resulted in a  $1.59 \pm 0.001$ -fold ( $P < 0.0001$ ) upregulation of plasmin generation, which is consistent with the role of p11 in the regulation of plasmin generation. This demonstrates the correlation between the upregulation of plasmin generation concomitant with the upregulation of p11, which are the result of the induction of PML/RAR $\alpha$  expression.

**Figure 40: Induction of PML/RAR $\alpha$  in PR9 cells upregulates the rate of plasmin generation and the expression of p11 and p36, but does not affect the expression of other plasminogen receptors.**

Cell lysates were prepared from PR9 cells were treated without or with 100  $\mu$ M zinc sulfate (ZnSO<sub>4</sub>) for 72 h. The expression of (A) PML-RAR $\alpha$ , p36 and p11 and (B) other plasminogen receptors (histone H2B, HMGB1, Plg-R<sub>KT</sub>,  $\alpha$ -enolase, and cytokeratin 8) were examined by western blot analysis with  $\beta$ -actin used as a loading control. Data is expressed as three independent experiments. (C) PR9 cells treated without or with 100  $\mu$ M zinc sulfate (ZnSO<sub>4</sub>) for 72 h were washed 3 times with Hanks balanced salt solution (HBSS containing 3 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>). The cells were resuspended in HBSS without or with 0.5  $\mu$ M glu-plasminogen. Cells were seeded to a 96-well plates (100, 000 cells per well) and incubated for 20–30 minutes before the addition of 500  $\mu$ M plasmin chromogenic substrate, S2251. The final volume was 200  $\mu$ L per well. Cleavage of S2251 was measured as absorbance at 405 nm for every 4 min over 4 h. The rate of plasmin generation was measured from the A405 nm vs. min<sup>2</sup> progress curves (n = 3). Data is expressed as 405 nm/min<sup>2</sup>/1 x 10<sup>6</sup> cells. Error bars represent the standard error of the mean (S.E.M.) obtained from three independent experiments with at least triplicate samples each. Statistical significance was determined using the Student t-test for unpaired observations, where \*\*\*\*P < 0.0001 is considered statistically significant. Western blot analysis of p11 and p36 was performed by Ryan Holloway, western blots for other plasminogen receptors and Dr. Alamelu (Dharini) Bharadwaj performed the plasmin generation assay.

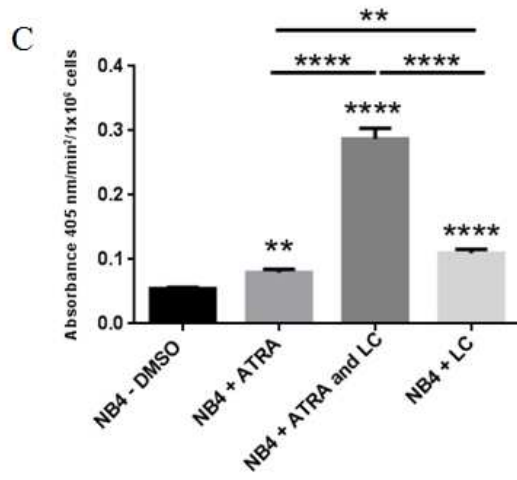
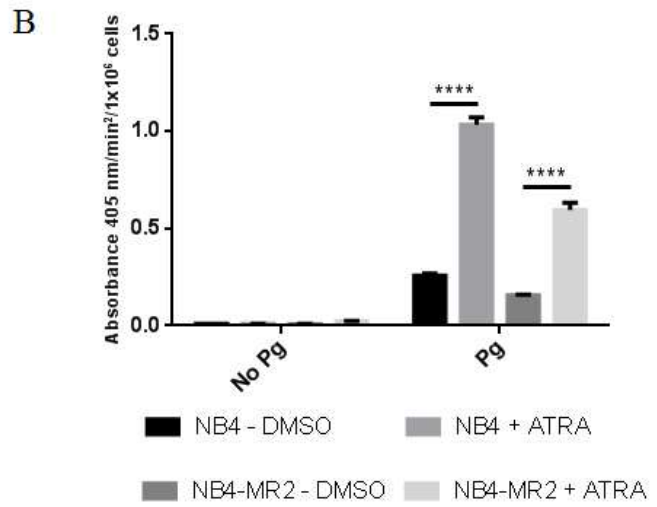
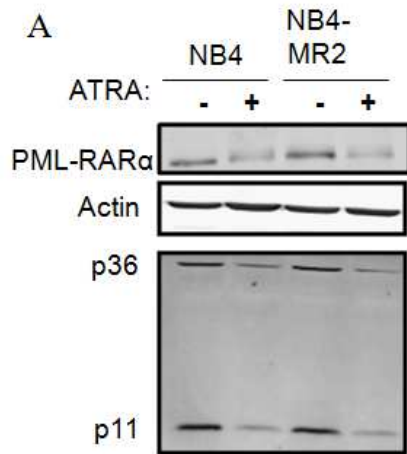


#### **4.1.2. ATRA treatment of NB4 and NB4-MR2 cells increases rate of plasmin generation**

ATRA is believed to correct the hyperfibrinolysis associated with APL by downregulation of p11 and reducing plasmin generation. O'Connell *et al.* previously showed that plasmin generation was decreased in NB4 cells after 72 h of ATRA treatment<sup>125</sup>. It is not certain whether the effect of ATRA alters plasmin generation by inducing granulocytic differentiation. Therefore, we next studied the effect of ATRA on the expression of PML/RAR $\alpha$ , p11 and p36, using NB4 cells or NB4-MR2 cells that are resistant to ATRA-induced differentiation<sup>275</sup>. ATRA treatment (1  $\mu$ M for 72 h) of NB4 cells and NB4-MR2 cells decreased p11 and p36 expression (Figure 41A). Plasmin generation was assessed in NB4 and NB4-MR2 cells with and without ATRA treatment. Surprisingly, plasmin generation was higher in the ATRA-treated NB4 cells by 4.00 $\pm$ 0.39-fold (P < 0.0001) and in NB4-MR2 cells by 3.79 $\pm$ 0.38-fold (P < 0.0001) compared to their respective control groups (Figure 41B). Plasmin generation was also assessed in NB4 cells treated with ATRA alone and/or with LC to prevent the ATRA-induced loss of p11 and p36. Because LC-treated cells did not survive at 72 h of treatment (Data not shown), we chose to assess plasmin generation at 48 h of treatment. Again, ATRA treatment of NB4 cells upregulated plasmin generation by 1.46 $\pm$ 0.01-fold (P < 0.01) and the combination of ATRA and LC further upregulated plasmin generation by 5.12 $\pm$ 0.02-fold (P < 0.0001) compared to the control group (Figure 41C). In addition, plasmin generation was upregulated in NB4 cells treated with LC alone by 2.01 $\pm$ 0.01-fold (P < 0.0001). These results indicate that ATRA stimulates plasmin generation independent of p11 or granulocytic differentiation.

**Figure 41: ATRA upregulates the rate of plasmin generation in NB4 and NB4-MR2 cells.**

Cell lysates were prepared from NB4 and NB4-MR2 cells treated with 1  $\mu$ M ATRA for 72 h. The level of (A) p11 and p36 was examined by western blot analysis with  $\beta$ -actin used as a loading control. Data is expressed as three independent experiments. (B) NB4 and NB4-MR2 cells treated with ATRA for 72 h and (C) NB4 cells treated with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M lactacystin for 48 h were washed 3 times with Hanks balanced salt solution (HBSS containing 3 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$ ). The cells were resuspended in HBSS without or with 0.5  $\mu$ M glu-plasminogen. Cells were seeded to a 96-well plates (100, 000 cells per well) and incubated for 20–30 minutes before the addition of 500  $\mu$ M plasmin chromogenic substrate, S2251. The final volume was 200  $\mu$ L per well. Cleavage of S2251 was measured as absorbance at 405 nm for every 4 min over 4 h. The rate of plasmin generation was measured from the A405 nm vs. min<sup>2</sup> progress curves (n = 3). Data is expressed as 405 nm/min<sup>2</sup>/1 x 10<sup>6</sup> cells. Error bars represent the standard error of the mean (S.E.M.) obtained from three independent experiments with at least triplicate samples each. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*\*P < 0.01 and \*\*\*\*P < 0.0001 are considered statistically significant. Western blot analysis of p11 and p36 was performed by Ryan Holloway, western blots of plasminogen receptors and the plasmin generation assays were performed by Dr. Alamelu (Dharini) Bharadwaj and Ryan Holloway.



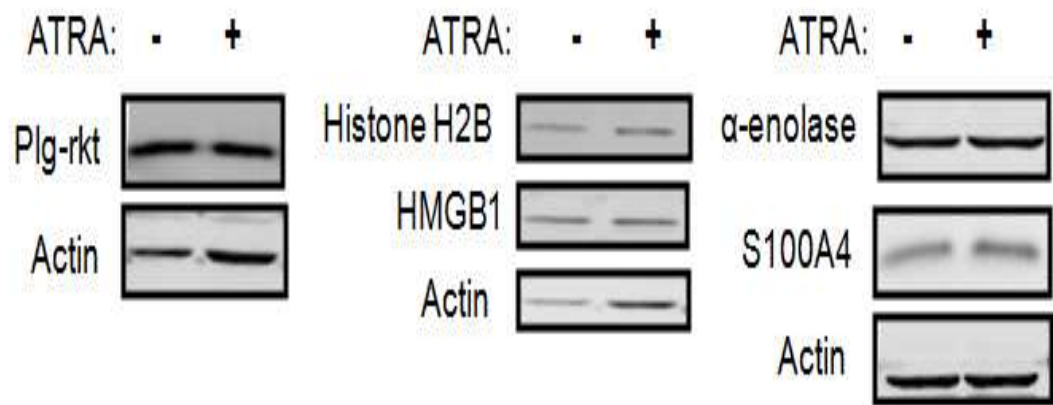
#### **4.1.3. Expression of plasminogen receptors in ATRA-treated NB4 cells**

It is not certain whether the upregulation of plasmin generation by ATRA treatment of NB4 cells affects the expression of other plasminogen receptors. The stimulation of plasmin generation by ATRA was surprising considering that ATRA treatment of NB4 cells also dramatically reduced p11 and p36 expression. Therefore, the expression of other plasminogen receptors was examined to determine if ATRA-dependent increase in plasmin generation was due to an increase of another plasminogen receptor. Western blot analysis showed that there were no changes in histone H2B, HMGB1, Plg-R<sub>KT</sub>, S100A4, and  $\alpha$ -enolase and no expression of cytokeratin-8 in the ATRA-treated NB4 cells compared to the DMSO vehicle control cells (Figure 42). This suggests the possibility that ATRA is stimulating plasmin generation of NB4 cells by inducing the expression of a novel plasminogen receptor, or plasminogen activators.

**Figure 42: ATRA does not affect of other plasminogen receptors in NB4 cells.**

Cell lysates were prepared from NB4 treated with 1  $\mu$ M ATRA for 72 hours. The level of plasminogen receptors (histone H2B, HMGB1, Plg-R<sub>KT</sub>,  $\alpha$ -enolase, and S100A4) was examined by western blot analysis with  $\beta$ -actin used as a loading control. Data is expressed as three independent experiments. Ryan Holloway prepared samples, and Dr. Alamelu (Dharini) Bharadwaj performed western blots for plasminogen receptors.





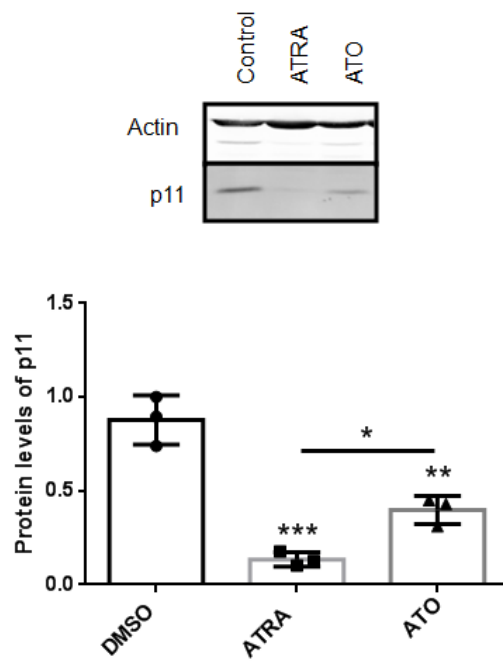
#### **4.1.4. Arsenic trioxide (ATO) treatment of NB4 cells downregulates plasmin generation concomitant with reduced p11 and p36 expression**

Like ATRA, arsenic trioxide (ATO) also corrects the hyperfibrinolytic disorder associated with APL. Hence, we assessed how ATO affected expression of p11 and other plasminogen receptors, and whether ATO treatment downregulates plasmin generation in NB4 cells. Protein levels of p11 were downregulated in ATRA-treated NB4 cells (1  $\mu$ M for 72 h) by  $7.69 \pm 0.04$ -fold and in ATO-treated NB4 cells (0.5  $\mu$ M for 72 h) by  $2.50 \pm 0.07$ -fold compared to the control (Figure 43A). Because ATRA treatment upregulated plasmin generation in NB4 cells, the effect of ATO was also assessed. We also determined whether ATO and ATRA treatment increased plasmin generation due to the loss of PML/RAR $\alpha$  or if the increase was an ATRA-dependent effect. Plasmin generation was downregulated by  $1.35 \pm 0.01$ -fold ( $P < 0.01$ ; Figure 43B) in the ATO-treated cells compared to the control group. This indicates that ATRA and ATO affect plasmin generation in NB4 cells through different mechanisms. Furthermore, the stimulation of plasmin generation observed with ATRA-treated NB4 cells suggests that this effect is attributed to retinoic acid signalling.

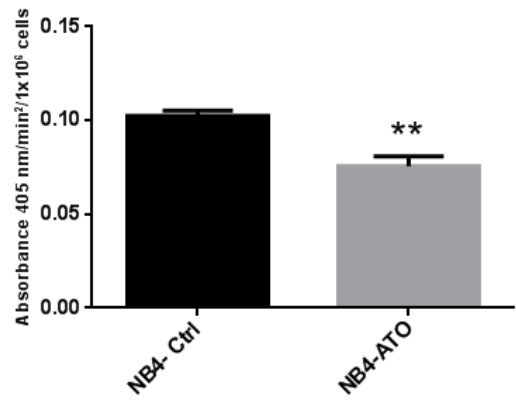
**Figure 43: Arsenic trioxide (ATO) downregulates the rate of plasmin generation concomitant with the downregulation of p11 and p36 expression in NB4 cells.**

(A) Cell lysates were prepared from NB4 cells treated with 0.5  $\mu\text{M}$  arsenic trioxide (ATO) for 72 h. The level of p11 and p36 was examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. (B) NB4 cells treated with ATO for 72 h were washed 3 times with Hanks balanced salt solution (HBSS containing 3 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$ ). The cells were resuspended in HBSS without or with 0.5  $\mu\text{M}$  glu-plasminogen. Cells were seeded to a 96-well plates (100,000 cells per well) and incubated for 20–30 minutes before the addition of 500  $\mu\text{M}$  plasmin chromogenic substrate, S2251. The final volume was 200  $\mu\text{L}$  per well. Cleavage of S2251 was measured as absorbance at 405 nm for every 4 min over 4 h. The rate of plasmin generation was measured from the A405 nm vs.  $\text{min}^2$  progress curves ( $n = 3$ ). Data is expressed as  $405 \text{ nm}/\text{min}^2/1 \times 10^6 \text{ cells}$ . Error bars represent the standard error of the mean (S.E.M.) obtained from three independent experiments with at least triplicate samples each. Statistical significance was determined using (A) one-way ANOVA (with Tukey multiple comparisons) or (B) the Student t-test for unpaired observations, where \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  are considered statistically significance.

A



B

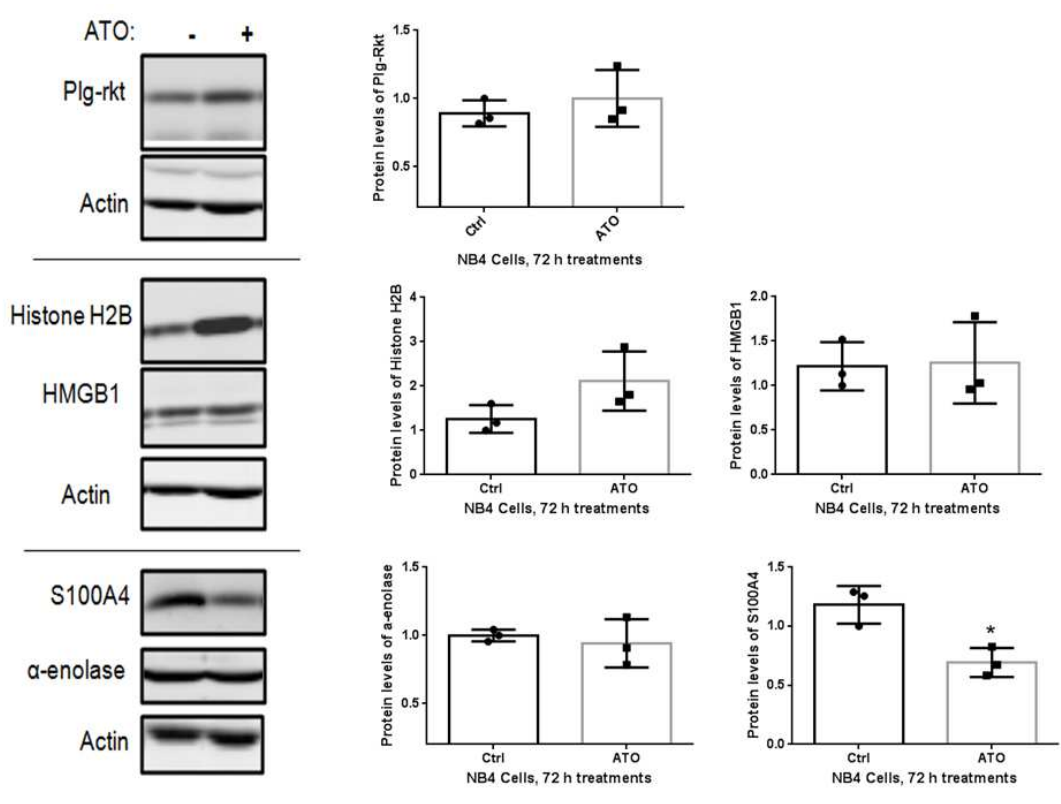


#### **4.1.5. Expression of plasminogen receptors in ATO-treated NB4 cells.**

The expression of other plasminogen receptors was assessed in ATO-treated NB4 cells to determine the involvement of other plasminogen receptors in the downregulation of plasmin generation. ATO treatment of NB4 cells did not change the expression of HMGB1, Plg-R<sub>KT</sub>, and  $\alpha$ -enolase. Although histone H2B was upregulated by ATO treatment, this increase was not statistically significant. However, we cannot rule out the possibility that this increase of histone H2B may be significant at the cell surface. Furthermore, ATO treatment of NB4 cells caused a decrease in S100A4 expression, which may contribute to the downregulation of plasmin generation (Figure 44). These results indicate that there are differences in the effects of ATRA and ATO on the expression of plasminogen receptors.

**Figure 44: ATO upregulates the expression of histone H2B, and downregulates the expression of p11, p36, and S100A4.**

Cell lysates were prepared from NB4 cells treated with 0.5  $\mu$ M for 72 h. The level of plasminogen receptors (histone H2B, HMGB1, Plg-R<sub>KT</sub>,  $\alpha$ -enolase, and S100A4) was examined by western blot analysis with  $\beta$ -actin used as a loading control. Data is expressed as three independent experiments. Ryan Holloway prepared samples, and Dr. Alamelu (Dharini) Bharadwaj performed western blots for plasminogen receptors.



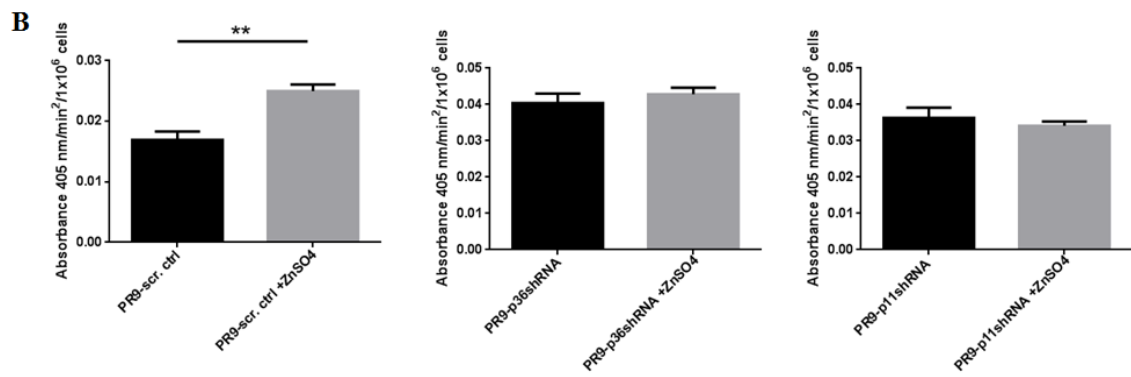
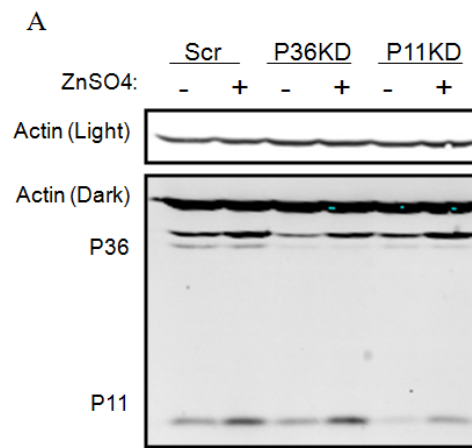
#### **4.1.6. Depletion of p11 or p36 prevents the upregulation of plasmin generation caused by PML/RAR $\alpha$ induction in PR9 cells**

To determine whether p11 and p36 contributed to the upregulation of plasmin generation in PML/RAR $\alpha$ -induced PR9 cells, plasmin generation was assessed in PR9 cells with p11 or p36 depleted by shRNA. To confirm the depletion, p11 and p36 expression was examined in the non-induced and induced PR9 cells stably expressing pSUPER.retro-scrambled control (scr. ctrl.), pSUPER.retro-p11-shRNA (p11-knockdown (KD)), or pSUPER.retro-p36-shRNA (p36-knockdown (KD)). Western blot analysis confirmed that p11 and p36 expression was downregulated in the PR9-p11-KD and PR9-p36-KD cell lines, respectively (Figure 45). The induction of PML/RAR $\alpha$  expression upregulated both p11 and p36 in all cell lines, yet p11 in the induced PR9-p11-KD cells was not upregulated to the level of p11 expression in the induced PR9-scr. ctrl cells. Similarly, p36 in induced PR9-p36-KD cells was not upregulated to the level of p36 expression in induced PR9-scr. ctrl cells. Induction of PML/RAR $\alpha$  upregulated plasmin generation in PR9-scr. ctrl cells by  $1.48 \pm 0.001$ -fold ( $P < 0.01$ ), but plasmin generation was not affected in the PR9-p11-KD or -p36-KD cell lines (Figure 45).



**Figure 45: The rate of plasmin generation does not increase due to PML/RAR $\alpha$  induction in p11- or p36-depleted PR9 cells.**

(A) Cell lysates were prepared from PR9 cells were treated without or with 100  $\mu$ M zinc sulfate (ZnSO<sub>4</sub>) for 72 h. The level of p11 and p36 was examined by western blot analysis with  $\beta$ -actin used as a loading control. Data is expressed as three independent experiments. (B) PR9 cells treated without or with 100  $\mu$ M zinc sulfate (ZnSO<sub>4</sub>) for 72 h were washed 3 times with Hanks balanced salt solution (HBSS containing 3 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>). The cells were resuspended in HBSS without or with 0.5  $\mu$ M glu-plasminogen. Cells were seeded to a 96-well plates (100, 000 cells per well) and incubated for 20–30 minutes before the addition of 500  $\mu$ M plasmin chromogenic substrate, S2251. The final volume was 200  $\mu$ L per well. Cleavage of S2251 was measured as absorbance at 405 nm for every 4 min over 4 h. The rate of plasmin generation was measured from the A405 nm vs. min<sup>2</sup> progress curves (n = 3). Data is expressed as 405 nm/min<sup>2</sup>/1 x 10<sup>6</sup> cells. Error bars represent the standard error of the mean (S.E.M.) obtained from three independent experiments with at least triplicate samples each. Statistical significance was determined using the Student t-test for unpaired observations, where \*\*P < 0.01 is considered statistically significant. Ryan Holloway performed western blot analysis of p11 and p36, and Dr. Alamelu (Dharini) Bharadwaj performed the plasmin generation assays.



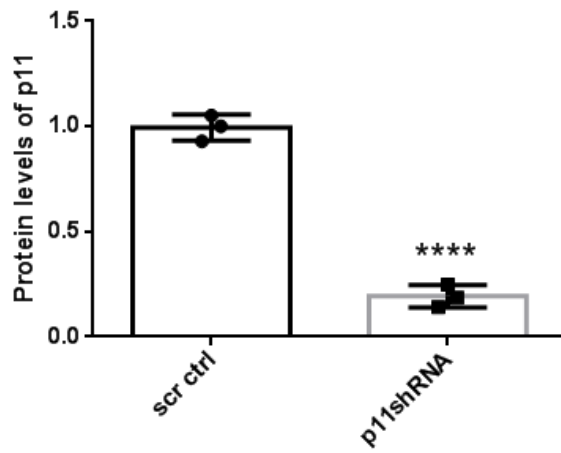
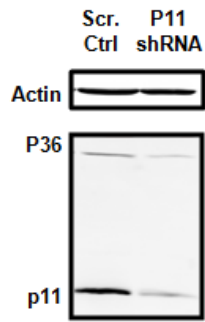
#### **4.1.7. Depletion of p11 from NB4 cells does not affect plasmin generation**

To determine the contribution of p11 in plasmin generation by NB4 cells, p11 expression and plasmin generation were assessed in NB4 cells stably transfected using pSUPER.retro-scrambled control (scr. ctrl.) or pSUPER.retro-p11-shRNA (p11-KD). The depletion of p11 was confirmed by western blot analysis that showed p11 expression was decreased by  $5.26 \pm 0.05$ -fold ( $P < 0.0001$ ) in NB4-p11KD cells compared to NB4-scr. ctrl. cells (Figure 46A). Next, plasmin generation was examined in NB4-scr. ctrl and NB4-p11KD cell lines. Plasmin generation was similar between the NB4-scr. ctrl and NB4-p11KD cells (Figure 46B), indicating that p11 does not contribute to the excessive production of plasmin associated with APL.

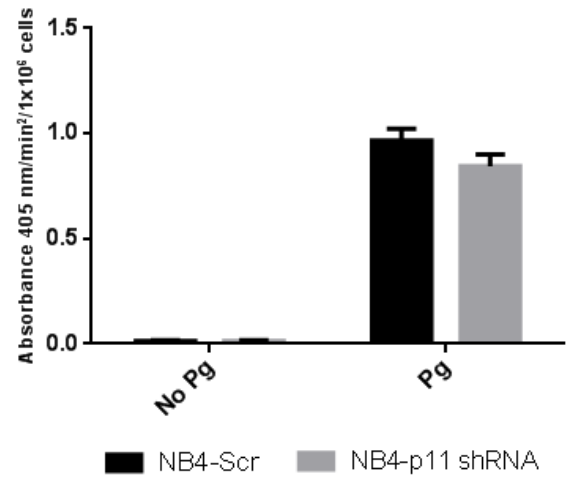
**Figure 46: The rate of plasmin generation is not affected by the depletion of p11 in NB4 cells.**

Cell lysates were prepared from NB4-pSUPER.retro-scrambled control (NB4-scr) and – p11shRNA (p11shRNA). (A) The level of p11 and p36 was examined by western blot analysis with  $\beta$ -actin used as a loading control. Quantification of protein levels by western blot analysis was performed as described in the methods section 2.11.2. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. (B) NB4-scr. and – p11shRNA cells were washed 3 times with Hanks balanced salt solution (HBSS containing 3 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$ ). The cells were resuspended in HBSS without or with 0.5  $\mu\text{M}$  glu-plasminogen. Cells were seeded to a 96-well plates (100,000 cells per well) and incubated for 20–30 minutes before the addition of 500  $\mu\text{M}$  plasmin chromogenic substrate, S2251. The final volume was 200  $\mu\text{L}$  per well. Cleavage of S2251 was measured as absorbance at 405 nm for every 4 min over 4 h. The rate of plasmin generation was measured from the A405 nm vs.  $\text{min}^2$  progress curves ( $n = 3$ ). Data is expressed as  $405 \text{ nm}/\text{min}^2/1 \times 10^6 \text{ cells}$ . Error bars represent the standard error of the mean (S.E.M.) obtained from three independent experiments with at least triplicate samples each. Statistical significance was determined using the Student t-test for unpaired observations. Ryan Holloway performed western blot analysis of p11 and p36, and Dr. Alamelu (Dharini) Bharadwaj and Ryan Holloway performed the plasmin generation assay.

**A** NB4 cells stably transfected with:



**B**



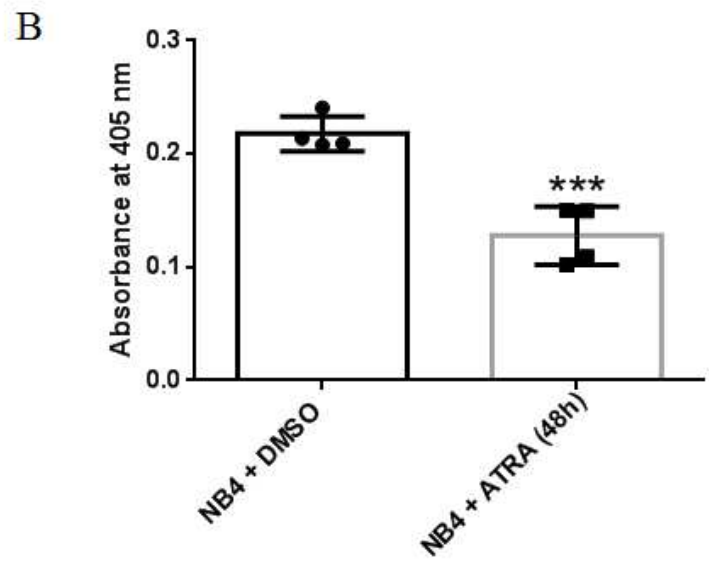
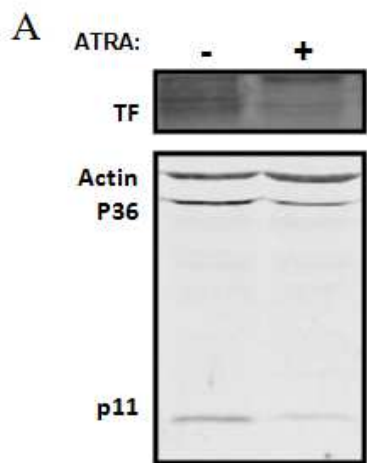
## **4.2. Regulation of procoagulant tissue factor (TF) by PML/RAR $\alpha$ and ATRA treatment of NB4 cells**

### **4.2.1. ATRA treatment of NB4 cells decreases expression and activity of TF**

APL is associated aberrant fibrinolytic activity, but is also associated with aberrant activation of coagulation due in part to elevated activity of tissue factor (TF)<sup>137</sup>, which is downregulated by ATRA treatment<sup>155</sup>. We previously demonstrated a role for p11 and p36 in regulating fibrinolysis in APL<sup>125</sup> and endothelial cells<sup>116</sup>. However, p11 and p36 may be also involved in the regulation of coagulation as recent studies showed that p36 regulates TF expression and activity in monocytes<sup>159,160</sup>. ATRA treatment should abrogate the coagulation activity of NB4 cells; hence, we assessed whether ATRA would downregulate TF expression and activity, which are responsible for blood coagulation. This will confirm whether the effect of ATRA on coagulation in APL cells is consistent with the findings from previous studies<sup>155,287</sup>. Western blot analysis shows a loss of TF expression in NB4 cells after a 48 h treatment with ATRA (Figure 47A). Furthermore, TF activity was significantly reduced by  $1.7 \pm 0.026$ -fold ( $P < 0.001$ ) in ATRA-treated NB4 cells compared to the control (Figure 47B).

**Figure 47: ATRA downregulated TF expression and activity in NB4 cells.**

Cell lysates were prepared from NB4 cells treated with 1  $\mu$ M ATRA for 48 h. (A) The level of tissue factor (TF), p11 and p36 was examined by western blot analysis with  $\beta$ -actin used as a loading control. Data is expressed as three independent experiments. (B) FVIIa and FX were added to total cell lysates (60  $\mu$ g of protein) and then TF activity was measured by the amount of FXa generated using a chromogenic FXa substrate. Absorbance of the chromogenic FXa substrate (37°C) was measured at 405 nm after 20 min. Data is expressed as mean  $\pm$  S.D. of four independent experiments. Statistical significance was determined using the Student t-test for unpaired observations, where \*\*\*P < 0.001 is considered statistically significant.



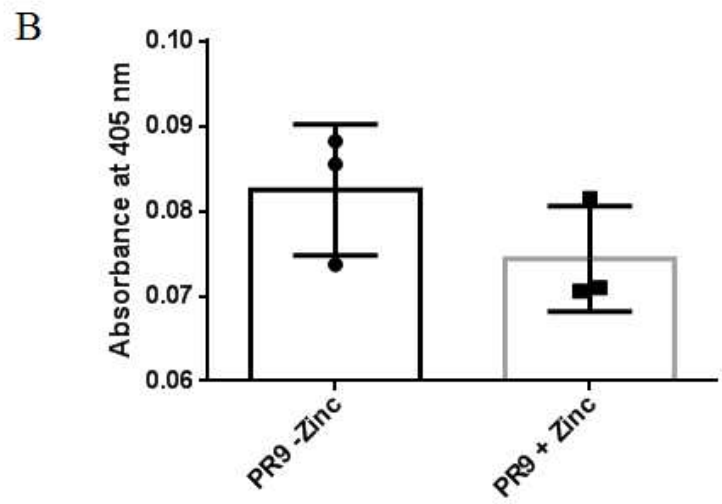
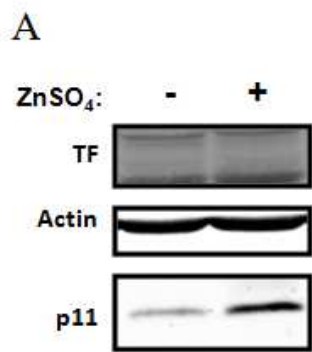


#### **4.2.2. Induction of PML/RAR $\alpha$ does not affect expression or activity of the TF**

Because recent studies discovered that p36 is involved in TF activity, we examined if the upregulation of p11 and p36 due to PML/RAR $\alpha$  induction in the PR9 cells would result in an increase of TF expression and/or activity. However, the induction of PML/RAR $\alpha$  expression in PR9 cells did not affect TF activity compared to the non-induced cells (Figure 48A). In addition, western blot analysis showed little to no detectable TF protein in the PR9 cells with or without PML/RAR $\alpha$  induction (Figure 48B). Notably, this may be attributed to low TF protein levels in unstimulated U937 cells<sup>288</sup>, a cell line from which the PR9 cells are derived.

**Figure 48: The induction of PML/RAR $\alpha$  in PR9 cells does not affect TF expression or activity.**

Cell lysates were prepared from PR9 cells treated with 100  $\mu$ M ZnSO<sub>4</sub> for 48 h. (A) The level of tissue factor (TF), p11 and p36 was examined by western blot analysis with  $\beta$ -actin used as a loading control. Data is expressed as three independent experiments. (B) FVIIa and FX were added to 60  $\mu$ g of total cell lysates (60  $\mu$ g of protein) and then TF activity was measured by the amount of FXa generated using a chromogenic FXa substrate. Absorbance of the chromogenic FXa substrate (37°C) was measured at 405 nm after 20 min. Data is expressed as mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using the Student t-test for unpaired observations.

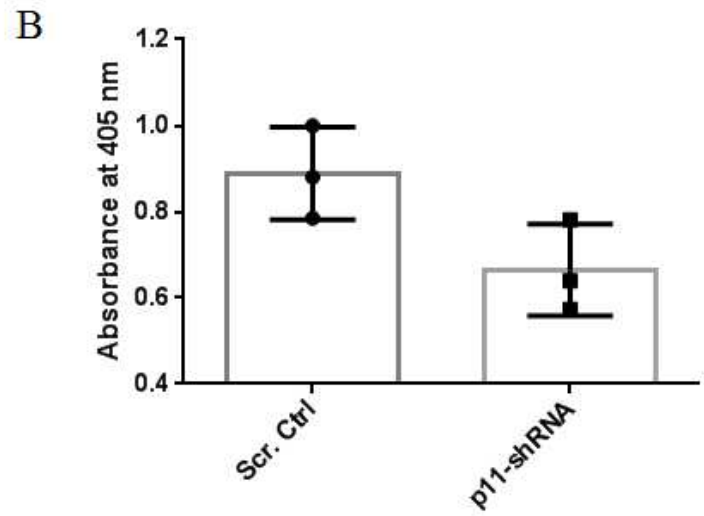


### **4.2.3. Depletion of p11 from NB4 cells does not affect the expression or activity of TF**

The depletion of p11 in NB4 cells was shown to decrease plasmin generation in APL cells, without changing total or surface p36 expression<sup>125</sup>, but we now showed that the loss of p11 does not affect plasmin generation. Although the contribution of p11 in fibrinolytic activity of NB4 cells was determined, the involvement of p11 in regulating TF expression, TF activity, and/or coagulation in APL was not investigated. In p11-depleted NB4 cells (NB4-p11shRNA), the deficiency of p11 had no effect on TF protein levels (Figure 49A). Furthermore, TF activity was not significantly reduced in NB4-p11shRNA cells compared to the NB4- scrambled control (NB4-scr.Ctrl) cells (Figure 49B).

**Figure 49: TF expression and activity are not affected by the depletion of p11 in NB4 cells.**

Cell lysates were prepared from NB4-pSUPER.retro-scrambled control (NB4-scr) and – p11shRNA (p11shRNA). (A) The level of tissue factor (TF), p11 and p36 was examined by western blot analysis with  $\beta$ -actin used as a loading control. Data is expressed as three independent experiments. (B) FVIIa and FX were added to 60  $\mu$ g of total cell lysates (60  $\mu$ g of protein) and then TF activity was measured by the amount of FXa generated using a chromogenic FXa substrate. Absorbance of the chromogenic FXa substrate (37°C) was measured at 405 nm after 20 min. Data is expressed as mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using the Student t-test for unpaired observations.



## **CHAPTER 5: DISCUSSION**

### **5.1. ATRA stimulates plasmin generation in APL cells independent of the AIIIt heterotetramer**

Excessive plasmin activity is believed to be one of the main mechanisms contributing to the life-threatening hemorrhagic disorder in APL patients<sup>134,142,154</sup>. Plasmin activity is regulated by a variety of extracellular factors and cell surface plasminogen receptors, but the only plasminogen receptor that is upregulated due to expression of the PML/RAR $\alpha$  oncoprotein in APL promyelocytes is S100A10 (p11). P11 is also highly expressed in other types of cancers<sup>60,110,117</sup> due in part by its upregulation by the oncoprotein, Ras. We previously demonstrated that depletion of p11 by shRNA or ATRA treatment of NB4 cells reduces plasmin binding, plasmin generation and invasiveness. A recent study<sup>286</sup> showed that the initial rate of plasmin generation in the presence of tPA, was inhibited in NB4 and zinc-induced PR9 cells using antibodies against p36, and to a lesser extent, p11. Furthermore, ATRA treatment resulted in a downregulation of plasmin generation in APL blasts and zinc-induced PR9 cells. Consistent with these studies, we showed that ATRA treatment decreased p11 and p36 expression in NB4 cells; however, we found that treatment ATRA stimulated plasmin generation. Our current findings showed that plasmin generation increased after 48 h and 72 h treatments with ATRA, which has been tested using NB4 cells obtained from different sources (DSMZ, Dr. Wilson Miller Jr., and NB4 cells used in O'Connell *et al*<sup>125</sup>). The contradictions between our current finding and these studies is likely due to the reduction of cell numbers used for the plasmin generation assay (1 x 10<sup>5</sup> cells compared to 1 x 10<sup>6</sup> cells per well of a 96-well plate). We have observed that the rate of

plasmin generation is lower when  $1 \times 10^6$  cells are seeded per well of a 96-well plate compared to  $1 \times 10^5$  cells (Appendix B, Figure S1). It is possible that the results of plasmin generation assays for ATRA-treated NB4 cells reported by other laboratories were inaccurate because an excessive number of cells were used in the plasmin generation assay. We have observed that in order to insure a linear relationship between cell number and plasmin activity, the number of cells to be used in the assay must be carefully determined. Hence, the reduction of cell number used in the assay produced results different from previous studies, indicating that plasmin generation by the NB4 or PR9 cells is affected by cell density. Additionally, to account for any error in estimating the number of cells used in the assay, cells were counted before and after the plasmin generation assay, and the data was normalized to cell numbers counted after the assay completion.

Although previous findings<sup>125,286</sup> are consistent with clinical observations that ATRA therapy reverses the coagulopathy associated with APL<sup>152</sup>, several studies found that ATRA treatment promotes the migration and invasiveness of NB4 cells, due in part to increased plasmin activation. This effect is associated with 1) uPA/uPAR-driven plasmin activity of NB4 cells<sup>225,289</sup>, 2) the involvement of MMP9<sup>225</sup> and 3) increased cell adhesion molecules (e.g. CD11B, CD18, CD29)<sup>290,291</sup>. The increases of adhesion molecules<sup>292</sup> and plasminogen activators<sup>226</sup> also corresponds to the clinical findings with ATRA treatment of APL patients. The results of O'Connell *et al* proposed that p11 was the critical regulator of plasmin generation in NB4 cells and depletion of p11 using ATRA or shRNA resulted in a significant decrease in plasmin generation. In the present study, ATRA treatment of NB4 cells dramatically increased plasmin generation, despite



the depletion of p11 levels. Furthermore, we found no increase in other plasminogen receptors to explain this effect, suggesting the involvement of other regulators of plasmin activity or a novel plasminogen receptor. Devy *et al.* demonstrated that a 3-day ATRA treatment increased uPA expression concomitant with increased invasiveness and migration of NB4 cells, which was greatly inhibited by the plasmin inhibitor aprotinin<sup>225</sup>. Both Zang *et al.*<sup>290</sup> and Mustjoki *et al.*<sup>289</sup> noted that ATRA treatment of NB4 cells produced a rapid and transient induction of uPA/uPAR, which could explain in part the increase of plasmin generation in ATRA-treated NB4 cells. Mustjoki *et al.* found that uPA protein increased on the cell surface and in conditioned media of NB4 cells after 24 h of ATRA treatment, and uPAR mRNA also increased at 46 h. Furthermore, plasmin generation was shown to be highest for the NB4 cells at 24 h of ATRA treatment compared to the control group, but plasmin generation then decreased to the control levels at 46 h of treatment. However, this study performed the plasmin generation assays by incubating cells with plasminogen for 30 min and then eluting cell-bound plasmin for enzymatic assays at several time points. Our method differs in that plasmin generation was assessed over the course of 4 h using the cells plated in 96-well plates. Furthermore, cells were pre-incubated in the absence or presence of plasminogen prior to the addition of the chromogenic plasmin substrate, as opposed to incubating cells briefly with plasminogen and assessing the plasmin generated at several time points. Mechanisms other than the upregulation of plasminogen activators have been found to contribute to enhanced plasmin activity in APL cells. Cao *et al.* recently demonstrated a role for cell-free DNA from APL promyelocytes in activating plasmin<sup>224</sup>. They found that ATRA induced the release of extracellular chromatin from APL promyelocytes resulting in

increased plasmin activity similar to our current data. Together, these findings demonstrate that ATRA-stimulated plasmin generation occurred independent of p11 and p36 due to other mechanisms such as increase in plasminogen activators and release of extracellular chromatin.

To determine if the increased plasmin generation by ATRA treatment was due to granulocytic differentiation or an ATRA-dependent effect, plasmin generation was assessed in 1) ATRA-resistant NB4-MR2 cells and 2) ATO-treated NB4 cells. We found that ATRA increased plasmin generation in NB4 and differentiation-resistant NB4-MR2 cells, but ATO treatment downregulated plasmin generation in NB4 cells, indicating that the increase in plasmin generation was ATRA-dependent. This result is consistent with *in vivo* findings<sup>226</sup> in which the haemostatic parameters of patients with APL treated with ATRA over 21 days showed that ATRA treatment resulted in increased tPA and decreased PAI-1 levels in the blood of patients with APL. In contrast, the blood of ATO-treated patients with APL showed a slight decrease of tPA levels and no change in PAI-1 levels. We also examined the effect of ATRA-induced plasmin generation in a non-APL cancer cell, MCF-7, where ATRA treatment resulted in the downregulation of plasmin generation, showing that ATRA can affect plasmin generation independent of the presence of the PML/RAR $\alpha$  oncoprotein. These findings demonstrate that ATRA stimulates plasmin generation independent of p11 in APL cells, but may reduce p11-dependent plasmin generation in other cancers.

## **5.2. P11 is dispensable in promoting plasmin generation in APL cells**

Since ATRA treatment of NB4 cells downregulated p11 and upregulated plasmin

generation, we addressed whether depletion of p11 by shRNA would result in the reduction of plasmin generation. The induction of PML/RAR $\alpha$  in PR9 cells was previously shown to increase plasmin generation and this effect was inhibited by depletion of p11<sup>125</sup> or in other studies by antibodies against p11 or p36<sup>286</sup>. In addition, we previously observed that p11-depleted NB4 cells showed a reduction in the rate of plasmin generation by 70% when cells were incubated with plasminogen, and this reduction was similar when cells were incubated with plasminogen and uPA<sup>125</sup>. Our current findings confirmed that shRNA-depletion of p11 or p36 blocked the upregulation of plasmin generation by PML/RAR $\alpha$ -induction of PR9 cells. However, depletion of p11 in NB4 cells downregulated plasmin generation slightly, but not significantly as previously demonstrated. Given these findings, it is possible that the contribution of p11 to plasmin generation differs in the PR9 and NB4 cell lines, and it is likely that multiple regulators of plasmin activity are contributing to the abnormal fibrinolysis in APL independent of p11.

### **5.3. P11 may not be involved in regulation of coagulation through modulation of tissue factor activity**

In addition to excessive fibrinolysis, abnormal activation of coagulation is also a major feature contributing to the life-threatening bleeding complications associated with APL<sup>135,155,156</sup>. For the first time, we examined the contribution of p11 in regulating TF activity in APL using NB4 cells depleted of p11. This aimed to determine whether the loss of p11 reduced TF activity or expression. P36 is implicated in regulating TF expression and activity in human monocytes as p36-depleted monocytes show decreased

TF expression and activity when TF expression was stimulated in the presence of anti-B2-glycoprotein I antibody<sup>159</sup>. In addition, depletion of p36 in murine monocytes show a dramatic reduction in TF expression<sup>160</sup>. A role of p36 in binding to and regulating FXa activity was also found to be involved in idiopathic pulmonary fibrosis<sup>161</sup>, further supporting p36 as a mediator of coagulation. It is known that p11 is necessary for the transport of p36 to the cell surface; hence, the loss of p11 may reduce the surface levels of p36. This suggests that the AII<sub>t</sub> heterotetramer is involved in the regulation of TF activity at the cell surface. We also examined TF expression and activity in ATRA-treated NB4 cells, and confirmed that ATRA treatment of NB4 cells reduced TF expression activity. As expected, ATRA also downregulated TF activity by ~50% in NB4 cells. Although ATRA induced the loss of p11 and p36 concomitant with the reduction of TF expression and activity, p11-depletion of NB4 did not affect TF expression. However, the reduction in TF activity observed in p11-depleted NB4 cells compared to control NB4 cells was not statistically significant. At this point, a role of p11 in regulating TF activity has not been established in the literature.

#### **5.4. P11 is a transcriptional target of retinoic acid receptors and may be upregulated in APL due to disruption of endogenous RAR $\alpha$ function due to PML/RAR $\alpha$**

In APL promyelocytes, PML/RAR $\alpha$  exerts a dominant-negative effect on endogenous RAR $\alpha$  by binding to retinoic acid response elements (RAREs) of RAR $\alpha$ -regulated genes<sup>134,136,154</sup>. ATRA treatment of APL promyelocytes stimulates PML/RAR $\alpha$  degradation and subsequently abolishes the dominant-negative effect that prevents

endogenous RAR $\alpha$  transcriptional activity<sup>134,154,294</sup>. We found that PML/RAR $\alpha$  is a major regulator of p11 and p36 protein expression in APL by demonstrating that (1) ATRA down-regulated PML/RAR $\alpha$ , p11 and p36 in NB4 cells independent of ATRA-induced differentiation and (2) induction of PML/RAR $\alpha$  in PR9 cells upregulated p11 and p36 protein expression. Our analysis of the transcriptional regulation of p11 and p36 revealed that induction of PML/RAR $\alpha$  also resulted in a significant increase in p11 and p36 mRNA. Furthermore, ATRA treatment of NB4 cells resulted in a dramatic decrease in p11 and p36 mRNA. This indicated that PML/RAR $\alpha$  directly regulates p11 transcriptionally and presumably post-translationally by activating the transcription and protein expression of p36. More specifically, PML/RAR $\alpha$  indirectly regulates p11 post-translationally by providing the newly translated p11 protein with its stabilizing binding partner p36. ATRA directly regulates p11 transcription possibly by blocking PML/RAR $\alpha$ -stimulated p11 transcription, and indirectly by blocking p36 transcription thus resulting in less p36 protein to stabilize p11 proteins. However, a direct effect of ATRA on p11 transcription in NB4 cells is possible since we observed a direct effect of ATRA on p11 transcription in MCF-7 cells, which do not express PML/RAR $\alpha$ . ATRA likely affects p11 transcription due to the presence of a RARE motif identified in the p11 promoter. A recent proteomics study has suggested that p11 and other members of the S100 protein family are regulated by retinoic acid signaling in MCF-7 cells<sup>285</sup>. In our study, our observations that the RAR $\alpha$  ligand ATRA downregulated p11 mRNA and protein levels in APL and non-APL cells, and that a RARE motif is predicted in the p11 promoter region also indicates that p11 is regulated by retinoic acid signalling. The effect of ATRA on p11 expression in APL and non-APL cells indicates that RAR $\alpha$  is a key

transcriptional regulator of p11 and the aberrant expression of p11 in APL is the result of disruption of normal RAR $\alpha$  function by PML/RAR $\alpha$ . Therefore, although our data shows that ATRA regulates p11 protein levels indirectly by both transcriptional and post-translation mechanisms, a direct effect on p11 transcription in NB4 cells is also possible. The action of LC is more complicated as it not only blocks the proteasomal degradation of p11 but also causes an increase in p11mRNA. The simplest explanation is that LC prevents the ATRA-stimulated proteasomal destruction of PML/RAR $\alpha$ . This would result in increased PML/RAR $\alpha$ -dependent stimulation of p11 and p36 transcription and increased expression of partnered p11 protein.

Previous findings showed that the depletion of PML/RAR $\alpha$  by ATRA treatment of NB4 cells or the induction of PML/RAR $\alpha$  expression in PR9 cells modulates p11 and p36 protein expression, but not transcripts levels. However, several groups found that ATRA treatment of NB4 cells reduced p11 and p36 mRNA levels<sup>146,277,295</sup>. The recent study by Huang *et al*<sup>296</sup> present that induction of PML/RAR $\alpha$  in PR9 cells transcriptionally upregulates p36, but not p11. However, the standard deviation for the p11 qPCR data was too large to adequately interpret the effect of PML/RAR $\alpha$  on p11 transcript levels. The discrepancy between our previous and present findings is likely due to improvements in methodology that included using the chloroform-phenol extraction method normalizing qPCR data to three reference genes instead of one.

### **5.5. ATRA can reduce p11 expression independent of the presence of PML/RAR $\alpha$ in non-APL cancers**

ATRA is one of the prominent chemotherapeutic agents used for APL treatment.

ATRA restores normal fibrinolysis and coagulation function by inducing PML/RAR $\alpha$  degradation and restoring granulocytic differentiation<sup>195</sup>. ATRA-induced PML/RAR $\alpha$  degradation causes a loss of p11 and p36 expression; however, it is not completely understood whether ATRA affects p11 and p36 expression in other cancer cell types independent of PML/RAR $\alpha$ . For example, ATRA treatment of the lung bronchial epithelial cell line, BEAS-2B, was shown to downregulate p11 protein levels after 5 days<sup>297</sup>. More recently, Jie *et al.* reported that ATRA treatment of dendritic cells isolated from mice resulted in decreased p11 and S100A4 mRNA levels<sup>298</sup>. Proteomic analysis showed that p11 and several other members of the S100 proteins are regulated by RAR $\alpha$  in which p11 expression was reduced in a tamoxifen-resistant MCF7-derived cells line with (LCC2) that had elevated expression of RAR $\alpha$  compared to parental MCF-7 cells<sup>285</sup>. Hence, we determined whether ATRA could affect p11 and p36 expression in MCF-7 and other non-APL cells, independent of PML/RAR $\alpha$ . Interestingly, we found that ATRA treatment of MCF-7 cells downregulated p11 transcript and protein levels without affecting p36 expression. In addition, ATRA treatment of triple-negative breast cancer cell lines, known to be resistant to the anti-tumourigenic effect of ATRA<sup>299,300</sup>, did not affect p11 or p36 levels. This presents p11 as a therapeutic target of ATRA in certain cancer types in addition to APL.

#### **5.6. Ubiquitin-independent proteasomal degradation is a novel mechanism regulating p11 stability.**

The life-threatening coagulopathy associated with APL is ameliorated primarily by treatment of patients with ATRA. Initially, ATRA reversal of the heightened

fibrinolytic activity of the leukemic promyelocytes was believed to be due in part by stimulating the degradation of the profibrinolytic protein p11. In order to improve our understanding of the mechanism by which ATRA affects the fibrinolytic activity of the leukemic promyelocytes, we observed that the ATRA-dependent loss of p11 was due to both transcriptional and post-translational mechanisms. Furthermore, contrary to the prevailing hypothesis, we observed that p11 protein was degraded by non-ubiquitin mediated proteasomal degradation when purified p11 protein was proteolyzed in vitro in the presence of purified 20S proteasome.

The prevailing dogma proposes that in the absence of p36, p11 is unstable due to rapid ubiquitylation on its carboxy-terminal lysines and this directs p11 to the proteasome for degradation. Multiple laboratories have observed that depletion or knockout of p36 results in a dramatic decrease in p11 protein levels<sup>83,90,92,301</sup>. Furthermore, we observed that depletion of p36 resulted in the loss of p11 protein, which was recovered with LC. Since the p11 recovered by LC in these cells did not contain detectable ubiquitylated-p11 and p11 was not present as higher molecular weight species, we propose that both ATRA and agents that cause the dissociation of the p11-p36 interaction results in the ubiquitin-independent proteasomal degradation of p11.

Our data presents a new model for the regulation of p11 protein in which p11 is degraded by ubiquitin-independent proteasomal degradation (Figure 50). This model is supported by our observation ubiquitin-conjugated p11 is undetectable in p11 immunoprecipitates from ATRA-treated NB4 cells incubated with LC, even though LC causes the accumulation of many ubiquitylated proteins under these conditions, and we easily detected these protein with our methodology. Furthermore, western blots of p11



revealed that when ATRA-treated NB4 cells were incubated with LC, p11 protein levels increased but ubiquitin-conjugated higher molecular weight species of p11 were not detectable. When treated with LC, p11 transcripts were not significantly increased, but an increased of p11 protein levels was observed (Appendix B, Figure S2). We also observed that the inhibition of protein ubiquitylation by the inhibitor of the E1 ubiquitin-activating enzyme, PYR-41 prevented the LC-dependent accumulation of ubiquitin-conjugated proteins. This result was consistent with observations by Yang *et al.*<sup>302</sup> in which they demonstrated that PYR-41 prevented the accumulation of ubiquitylated proteins caused by LC treatment, but PYR-41 treatment alone did not affect the basal levels of ubiquitylated protein since not all ubiquitylation protein are targeted to the 26S proteasome for degradation. The prevailing data supporting the ubiquitin-mediated proteasomal degradation of p11 originated from studies in which both ubiquitin and p11 were co-overexpressed in HEK293T cells. These cells are amenable to overexpression experiments due to very low intracellular levels of p11 and p36 protein. He *et al.*<sup>90</sup>, showed that ubiquitylation was likely to involve Lys92 or Lys94 of the p11 carboxy-terminal sequence, <sup>89</sup>VHMKQKGKK<sup>97</sup>. This was derived from the overexpression of a series of carboxy-terminal deletions of p11 and the observation that loss of carboxy-terminal residues blocked ubiquitylation of p11. However, our data utilized mass spectrometric analysis of ubiquitylated-p11 and identified Lys57 as the primary site of ubiquitylation. Interestingly, Lys57 is a highly conserved and surface-exposed lysine that is conserved in all 16 species sequenced<sup>73</sup>. Furthermore, mapping of endogenous putative ubiquitylation sites in murine tissues by mass spectrometry identified Lys47, Lys54 and Lys57 as the primary ubiquitylated lysines in p11, although these sites were not verified

by site-directed mutagenesis. Our study showed that mutagenesis of Lys57 prevented the ubiquitylation of overexpressed p11, which indicated that this is the key site for p11 ubiquitylation. However, we cannot rule out the possibility that other ubiquitylation sites exist and play a role in the structure or function of p11. Despite our inability to detect ubiquitylated p11 in cells other than cells engineered to overexpress p11, we cannot rule out the possibility that ubiquitylated p11 plays an important role in cellular functions under certain physiological conditions.

We cannot rule out the possibility that p11 was de-ubiquitylated during cell lysis and SDS-PAGE analysis; however, this would be inconsistent with our ability to detect ubiquitylated-p11 when p11 and ubiquitin were overexpressed in HEK293T cells. Furthermore, EDTA present in our cell lysis buffer removes heavy metal ions necessary for deubiquitylase activity<sup>303</sup>. We attempted to use the deubiquitylase inhibitor, N-Ethylmaleimide (NEM), to prevent any removal of ubiquitin from p11, and this still did not allow visualization of ubiquitin-conjugated p11 species (Appendix B, Figure S3). We observed that the expression of the Lys57→Arg mutant of p11 (K57R) was similar to p11-wildtype (WT) and other lysine mutants of p11 suggesting that the loss of the ubiquitylation site of p11 did not prevent p11 degradation. We cannot exclude the possibility that there may be other non-ubiquitin modifications of Lys57 involved in regulating p11 protein stability. Other non-proteasomal mechanisms may regulate p11 protein stability; however, we tested various inhibitors to lysosomal degradation and calpain proteolysis, but these failed to prevent the degradation of p11. He *et al.*<sup>90</sup>, examined the effect of the Lys29 and Lys48 polyubiquitin linkages that are the predominate polyubiquitin linkages that direct proteins for proteasomal degradation.

Here, they used ubiquitin mutants where either Lys29 or Lys48 of ubiquitin was mutated to arginine (ub-K29R and ub-K48R). HEK293T cells co-expressing p11 and ub-K29R or ub-K48R resulted an increased of p11 levels. The flaw in this experiment is that they did not compare the effect of these mutant ubiquitins to wildtype ubiquitin to determine if wildtype ubiquitin also increased p11 expression. Our data showed that the loss of Lys48 in ubiquitin significantly increased p11 expression. When p11 was co-expressed with ub-K0 or ub-K48R, p11 expression increased significantly compared to cells co-transfected with p11 and ub-WT. We also observed that p11 co-expressed with the ub-K48 only mutant that lacks Lys29, did not increase p11 expression compared to cells co-transfected with p11 and ub-WT, indicating that Lys29 polyubiquitin linkages do not affect p11 protein levels. Furthermore, the co-expression of p11 and ub-WT did not show a difference to cells HEK293T cells overexpression p11 alone. These results show that if p11 is ubiquitylated, it may be directed for proteasomal degradation by Lys48-polyubiquitin linkages. However, we cannot rule out the possibility that the ubiquitin mutant lacking Lys48 cause the upregulation of p11 by an indirect effect as preventing Lys48-polyubiquitin linkages can have a global effect on proteins that may affect p11 by transcriptional and/or post-translational mechanisms. For example, it is possible that ub-K0 blocks global ubiquitylation resulting in the accumulation of a transcription factor that regulates p11 protein levels.

Although proteasomal inhibition by LC is often believed to block ubiquitin-dependent proteasomal degradation, it is important to stress that LC inhibits both ubiquitin-dependent and -independent mechanisms by targeting the catalytic  $\beta$ -subunits of the 20S proteasome core<sup>304</sup>. We observed that LC prevented the loss of p11 protein,

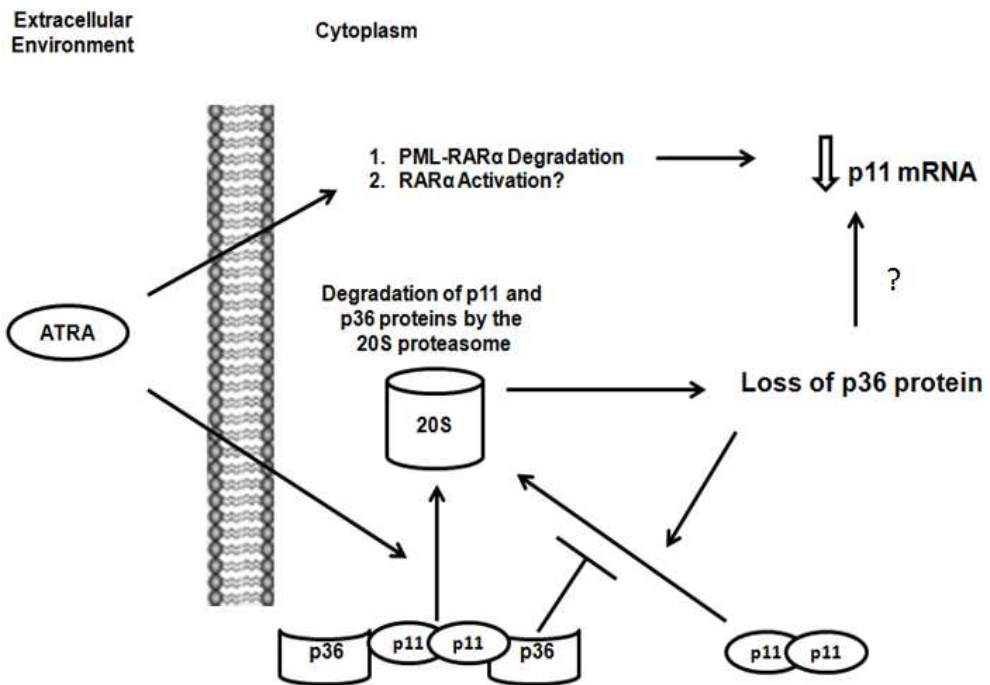
but inhibition of ubiquitylation by PYR-41 did not, suggesting that ubiquitylation of p11 is not required for its degradation by the proteasome. However, we cannot rule out the possibility that E1 ubiquitin-activating enzymes that are not inhibited by PYR-41 participate in the regulation of p11 levels. In contrast to LC, transfection of cells with ub-K0 should only result in inhibition of the degradation of proteins by the 26S proteasome. Interestingly, when HEK293T cells were transfected with ub-K0 alone, basal p11 levels did not increase. It was only after both ub-K0 and p11 were transfected that we observed p11 levels were higher than those observed for p11 and ub-WT. We favor the interpretation that ub-K0 caused an increase in overexpressed p11 by blocking ubiquitin-mediated 26S proteasomal degradation that was directed at only overexpressed p11. Therefore, we investigated whether p11 was degraded by the proteasome in an ubiquitin-independent manner by incubating purified p11 protein with the 20S proteasome, and we observed that it was rapidly degraded *in vitro*. The presence of intrinsically disordered regions is also a critical feature of proteins degraded in a ubiquitin-independent manner by the 20S proteasome, such as p53 and ornithine decarboxylase (Reviewed in <sup>305,306</sup>). However, disordered regions are also involved in regulation of ubiquitin-dependent proteasomal degradation (Reviewed in <sup>307</sup>). Disordered regions contain short linear motifs, called degrons, which are required for the recognition by E3 ubiquitin-ligases for ubiquitylation. Furthermore, the disordered regions also contain lysines targeted for ubiquitylation that are located in close proximity to the E3 ubiquitin-ligases binding motif; however, phosphorylation of the substrate is typically required in order for the E3 ubiquitin-ligase to recognize and bind to substrates (Reviewed in <sup>307</sup>). Intrinsically disordered regions have been identified in p11 as well as other members of the S100

family of proteins<sup>308</sup>, and Lys57 is located in the helix III region, which is an intrinsically disordered region. This suggests that the presence of an intrinsically disordered region around Lys57 of p11 may be critical in regulating both ubiquitin-independent and -dependent proteasomal degradation.

Phosphorylation of monomeric p36 by PKC was proposed to prevent the interaction between p11 and p36<sup>83,96</sup>. This was demonstrated in plasmin-treated human endothelial cells, where plasmin-dependent cleavage of cell surface-p36 causes it to associate with toll-like receptor 4 (TLR4), which then stimulates activity of conventional PKC (cPKC) isoforms ( $\alpha$ ,  $\beta$ )<sup>83</sup>. The cPKCs phosphorylate cytoplasmic p36 on serines 11 and 25, thus preventing the p11-p36 interaction and causing unpartnered p11 to be vulnerable to proteasomal degradation. Because ATRA is known to activate several PKC isoforms (conventional and novel)<sup>129,278</sup>, we examined if ATRA induced PKC activity in NB4 cells and whether this contributed to p11 degradation. Although the increase of phosphorylated PKC-substrates correlated with the loss of p11, inhibition of several different PKC isoforms failed to prevent p11 and p36 downregulation in ATRA-treated NB4 cells. Furthermore, immunoprecipitation of p36 from ATRA-treated NB4 cells showed that p36 was serine-phosphorylated to the same degree in both ATRA and control treatments. This indicated that ATRA-induced PKC activity does not disrupt the formation of the AII<sub>t</sub> heterotetramer by p36 phosphorylation on Ser11 and Ser25 as per the previously established model depicting the regulation of the p11-p36 interaction by PKC<sup>83</sup>. These findings reveal a novel mechanism of promoting p11 degradation independent of ubiquitylation and serine-phosphorylation of p36, distinct from plasmin-treated human endothelial cells<sup>83</sup>.

**Figure 50: Model of ATRA-dependent degradation of p11 by the 20S proteasome**

ATRA induces the loss of p11 expression by downregulation of mRNA levels and by stimulating p11 protein degradation by the proteasome. In APL cells treated with pharmacological doses of ATRA (1  $\mu$ M), ATRA binds to PML/RAR $\alpha$  and stimulates its destruction, resulting in the downregulation of p11 mRNA levels. In non-APL cells, ATRA binds to RAR $\alpha$  to stimulate its transcriptional functions that mediate the downregulation of p11 mRNA levels by either direct or indirect effects. Freshly translated p11 is only stable after forming a complex with p36. ATRA induces the degradation of both p11 and p36 proteins by the 20S proteasome in an ubiquitin-independent manner. The loss of p36 protein results in unpartnered p11 protein that is rapidly degraded by the 20S proteasome and possibly results in a loss of p11 mRNA.



### **5.7. P36 regulates p11 protein levels, but may regulate p11 transcript levels in certain cases.**

In the absence of p36, the p11 protein is ubiquitinated and directed to the proteasome for degradation, but multiple laboratories have observed that p11 transcripts are not affected by the depletion or knockout of p36<sup>91,94</sup>. Interestingly, in macrophages isolated from p36<sup>-/-</sup> mice, we found that there is a loss of p11 protein levels, yet LC and PYR-41 are unable to recover p11 expression. This suggested the possibility that p36 may play a role in stabilizing p11 transcripts. A RNA-binding motif has been identified in domain IV of p36<sup>79</sup> and has been found to mediate the binding to the 3'-untranslated regions (3'UTR) in the mRNA of *c-myc*<sup>78,309</sup>, *p53*<sup>310</sup>, *p36*<sup>311</sup>, *N-methyl-D-aspartate R1 (NMDAR1)*<sup>312</sup>, and *collagen propyl 4-hydroxylase- $\alpha$ (I) (c-p4h- $\alpha$ (I))*<sup>313</sup>. So far, p36 has been shown to bind to the 3'-UTR of several genes, but it is not certain that this would be the case if p36 were capable of binding to p11 mRNA. Our data indicates that the overexpression of p36 in HEK293T cells did not affect p11 mRNA, yet other groups have shown that the loss of p36 protein levels reduced p11 transcript levels in MDA-MB-435 human breast cancer cells<sup>301</sup> and L5178Y murine lymphoma cells<sup>314</sup>. Hence, we cannot rule out the possibility that p36 is capable of regulating p11 transcription. Considering our observations of ATRA-treated NB4 cells, we do not know if the loss of p11 transcripts may be attributed to the loss of p36 protein levels, the loss of PML/RAR $\alpha$ , or another protein associated with p36 and/or PML/RAR $\alpha$ . Likewise, upregulation of p36 protein levels in PML/RAR $\alpha$ -induced PR9 cells could also be responsible for the upregulation of p11 at the transcriptional and post-translational levels. Interestingly, ATRA treatment of MCF-7 cells resulted in the reduction of p11 transcripts and protein



levels without affecting p36. These data indicate that ATRA may be affecting p11 transcription and protein levels independent of p36 protein levels in MCF-7 cells.

### **5.8. Pitfalls of this study**

As part of the AIIIt heterotetramer complex, p11 is one of the major plasminogen receptors in numerous cells types. In this study, the effect of the PML/RAR $\alpha$  oncoprotein and ATRA treatment were shown to alter p11 total cellular levels and plasmin generation, but the evidence for p11 localization on the cell surface is lacking in this study. One of the previous studies by our laboratory has shown that p11 is detected at the cell surface of NB4 cells and zinc-induced PR9 cells using cell surface biotinylation and FACS analysis. Although attempts were made to assess p11 expression at the cell surface of NB4 cells, it was not detectable by cell surface biotinylation and FACS analysis. However, p11 was detectable on the cell surface of the lung carcinoma cell line A549, which abundantly expresses p11, using cell surface biotinylation (Appendix B, Figure S4). Hence, our inability to detect surface levels of p11 in NB4 cells may be due to a low level of p11 expression. Secondly, we have observed that p36 is not detected when p11 is immunoprecipitated from NB4 or PR9 cells. Likewise, p11 is not detected when p36 is immunoprecipitated from NB4 cells. We speculated that this might be attributed to the antibodies disrupting the p11-p36 interaction. For this reason, immunoprecipitation was performed using purified AIIIt heterotetramer isolated from bovine lung to determine if immunoprecipitated p36 would be associated with p11. Surprisingly, there was very little p36 associated with the immunoprecipitated p11 (Figure 21), suggesting the possibility that p11 and p36 antibodies may disrupt the p11-p36 interaction. Therefore, if these

antibodies disrupt the p11-p36 interaction, then this would complicate detection of p11 and p36 at the cell surface using FACS. Interestingly, Zobiack *et al.* also examined whether immunoprecipitated p11 was associated with p36, *vice versa*<sup>315</sup>. They observed that immunoprecipitated p11 was not associated with a large amount of p36 protein, and immunoprecipitated p36 was not associated with a large amount of p11. They reasoned that the AII<sub>t</sub> heterotetramer might have masked an epitope on p11 and p36 to which the antibodies would bind to their respective proteins.

In figure 46, the depletion of p11 and p36 attenuated the upregulation of plasmin generation in PML/RAR $\alpha$ -induced PR9 cells. The level of p11 protein also increased in PML/RAR $\alpha$ -induced PR9-p11KD cells, although they were lower than the PR9-scr. control cells. Likewise, the level of p36 protein also increased in PML/RAR $\alpha$ -induced PR9-p36KD cells, yet they were lower relative to the PR9-scr. control cells. Even though p11 or p36 expression was reduced, the induction of PML/RAR $\alpha$  still upregulated p11 and p36 protein levels without affecting plasmin generation. Here, the cell surface levels of p11 and p36 may be affected in either the PR9-p11KD or PR9-p36KD cells. It may be possible that the reduction of p11 or p36 in the PML/RAR $\alpha$ -induced PR9 cells may hinder the localization of the AII<sub>t</sub> heterotetramer to the cell surface.

## **CHAPTER 6: CONCLUSIONS**

### **6.1. Conclusion**

These findings demonstrate that ATRA therapy promotes the downregulation of p11 at the transcriptional and post-translational levels in APL and non- APL cells. We present evidence that ATRA induces ubiquitin-independent degradation of p11; a novel mechanism that contradicts the widely accepted paradigm that p11 is regulated by ubiquitin-dependent proteasomal degradation. Although ubiquitylated p11 is not detectable in NB4 or PR9 cells, co-overexpression of p11 and ubiquitin in HEK293T cells produced the ubiquitylation of p11 at Lys57 , yet we cannot rule out the possibility that p11 is ubiquitylated at this site under other physiological circumstances. The observation that ATRA affected p11 expression in non-APL cancer cells identifies p11 as a potential RAR $\alpha$  target gene, which may lead to targeting p11 in other cancers using retinoid therapy.

This study demonstrated that the hyperfibrinolysis associated with APL promyelocytes may not depend entirely upon p11-dependent plasmin generation. Furthermore, plasmin generation by NB4 cells was affected differently using the treatments that cure APL. Treatment with either ATO or ATRA resulted in the depletion of p11 in NB4 cells with little to no effect on other plasminogen receptors, yet plasmin generation is downregulated by ATO and upregulated by ATRA treatment, which may indicate ATRA upregulates other regulators of plasmin activity. We also found that ATRA reduced p11 expression concomitant with reduced plasmin generation in non-APL cancer cells. This demonstrates p11 as a molecular target of ATRA therapy in RA-sensitive cancers other than APL.

## 6.2. Future Directions

Although we did not observe any change in p11 mRNA levels due to the overexpression of p36 protein in HEK293T, other groups have demonstrated that the loss of p36 protein levels reduced p11 transcript levels in human<sup>301</sup> and murine cells<sup>314</sup>. There are several possible mechanisms by which p36 could bind to p11 mRNA to regulate its stability (Reviewed in<sup>316,317</sup>): (1) preventing the deadenylation of the poly-A tail, (2) binding to *cis*-acting regulatory elements of the 3'- or 5' untranslated region (UTR) or the mRNA coding region or (3) by affecting the localization of the p11 mRNA. Deadenylation of the poly-A tail, which protects mRNA from degradation, is the primary step in the degradation of most mRNAs (Reviewed in<sup>316,317</sup>). The poly-A-binding protein (PABP) complex protects mRNA from degradation by ribonucleases by binding the poly-A tail of mRNA<sup>316</sup>. It may be possible that p36 protects p11 mRNA from degradation by ribonucleases directly or by regulating PABP. There are various elements in the 3'-UTR, 5'-UTR and the mRNA coding regions in the mRNA of various genes that regulate their stability. The 3'-UTR of can contain *cis*-acting regulatory elements, such as AU-, GU-, CU-, or CA-rich elements, that bind RNA-binding proteins, or *trans*-acting regulatory elements (Reviewed in<sup>317</sup>). The 3'-UTR of  $\beta$ -adrenergic receptor mRNA, for example, binds the p37 AUF1 isoform that destabilizes its transcript<sup>318</sup>. The 5'-UTR of Il-2 mRNA contains a JNK-response element (JRE) that is responsible for stabilizing the transcript by binding nucleolin and YB-1<sup>319</sup>. The mRNA coding region of c-fos contains two coding region determinants (CDR-1 and CDR-2) that stabilize its mRNA by recruitment of a protein complex, consisting of PABP, the PABP-binding protein PAIP-1, the purine-rich RNA binding protein Unr, NSAP1, the p37 isoform of AUF1<sup>320-322</sup>. Exon 3 of c-myc

also contains a CDR that binds a CDR-binding protein that protects c-myc mRNA from endonucleases<sup>323,324</sup>. Localization of mRNA to free or membrane-bound ribosomes has been shown to affect immunoglobulin heavy-chain (IgG-Hc) mRNA stability, which depends on its localization to membrane-bound ribosomes during B-cell differentiation<sup>325</sup>. IgG-Hc mRNA stability was shown to depend on its localization to membrane-bound ribosomes as deletion of a signal sequence in IgG-Hc mRNA resulted in its translation by free ribosomes and unstable IgG-Hc mRNA during B-cell differentiation. So far, p36 has been shown to bind to the 3'-UTR of several genes, but it is not certain that this would be the case for p11 mRNA. It may be possible that p36 regulate p11 mRNA levels indirectly by affecting other protein that regulate p11 mRNA.

The observation that ATRA treatment of MCF-7 cells can downregulate p11 transcript and protein expression, without affecting p36, indicates that ATRA affects p11 expression by mechanisms independent of its effect on PML/RAR $\alpha$ . Since MCF-7 cells harbour RAR $\alpha$ , a transcription factor and receptor of ATRA, this suggests that retinoic acid signalling mediates p11 transcriptionally. Furthermore, several RAR $\alpha$ / $\gamma$ -binding motifs were predicted within the 10 kb region of the p11 promoter. If RAR $\alpha$ / $\gamma$  directly regulates p11 transcription, then it should be associated with the p11 promoter when examined using chromatin immunoprecipitation (ChIP). Understanding the mechanism of how retinoic acid signalling regulates p11 would assist in identifying cancers that are susceptible to agents such as ATRA that promote the loss of p11 and the p11-dependent plasmin generation associated with metastasis.

To our surprise, ATRA treatment stimulated plasmin generation of NB4 cells.

For this assay, we seeded 10-fold less cells than the investigation by O'Connell *et al.*<sup>125</sup>. Since we observed that plasmin activity was not measured accurately when the number of cells in the assay exceeded  $1 \times 10^5$  cells, we concluded that the previous results were in error. In addition, we observed no changes in the expression of other plasminogen receptors of ATRA-treated NB4 cells. However, we cannot discount the possibility of the existence of a novel plasminogen receptor whose expression is induced by ATRA treatment. This can be identified using the technique of plasminogen binding with a modified version of plasminogen containing a biotin ligase tag referred to as biotin identification (BioID)<sup>326</sup>. The BioID tagged plasminogen would to bind to plasminogen receptors and plasminogen receptors that are bound to the BioID tagged plasminogen could then be isolated using conventional methods to capture biotin. The plasminogen receptors isolated can be analyzed by mass spectrometry to determine if a novel plasminogen receptor is present on NB4 cells.

Finally, this study identified the primary site of ubiquitylation of p11 as Lys57. Furthermore, HEK293T cells co-expressing p11 and ubiquitin produced ubiquitylated p11, but this was blocked by the loss of the Lys57 residue of p11 or by co-expressing p36 with p11 and ubiquitin. The effect of ubiquitylation of p11 did not appear to support its degradation by the 26S proteasome, but ubiquitin can also mediate the localization of proteins and protein-protein interactions. An effect of ubiquitylation on modulating p11 localization has not been described. Zobiack *et al.* investigated the effect p36 had on p11 localization using live cell imaging of fluorescently tagged p11 and p36 in HepG2 cells, which express low levels of endogenous p11 and p36<sup>315</sup>. They observed that unpartnered p11 is localized to the cytoplasm, but p11 complexed to p36 is localized to the plasma

membrane. A similar approach could be used in which the effect of p11 ubiquitylation can be examined using live cell imaging of HEK293T cells expressing fluorescently tagged p11 and ubiquitin.

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## **APPENDIX A: MANUSCRIPT**

This Appendix contains the first publication generated from the work contained in this thesis. The manuscript has been submitted for publication in the journal *of Biological Chemistry* on April 2<sup>nd</sup>, 2018. Manuscript references have not been included.

Regulation of cell surface protease receptor S100A10 by retinoic acid therapy in acute promyelocytic leukemia (APL)

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\*Running title: *Ubiquitin-independent S100A10 degradation in APL*

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**Key Words:** S100A10; annexin A2; retinoic acid; APL; proteasome; ubiquitin.

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**ABSTRACT**

Acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia, is characterized by expression of the promyelocytic leukemia-retinoic acid receptor (PML/RAR $\alpha$ ) oncoprotein and life-threatening hemorrhagic complications due to excessive production of the fibrinolytic protease plasmin (Pm). We previously demonstrated that a prometastatic cell surface regulator of Pm generation, S100A10 (p11) is overexpressed in APL promyelocytes. Furthermore, treatment of APL promyelocytes with all-trans retinoic acid (ATRA) mitigated the hemorrhagic disorder by promoting the degradation of PML/RAR $\alpha$  and p11. Here, we show ATRA treatment of APL cell line, NB4, and MCF-7 breast cancer cells both reduced p11 transcript and protein levels suggesting that ATRA can regulate p11 levels independent of PML/RAR $\alpha$ . Lactacystin reversed the ATRA-dependent loss of p11, but did not increase ubiquitylation of p11

suggesting that ATRA promoted the proteasomal degradation of p11 in an ubiquitin-independent manner. The forced expression of ubiquitin and p11 in 293T cells resulted in ubiquitylation of p11 that was blocked by mutagenesis of lysine 57. Furthermore, proteasomal inhibition did not recover p11 levels in the absence of p36 protein levels. Overexpression of p36 upregulated p11 protein but not mRNA levels, indicating that p36 affects p11 post-translationally. This study highlights the complex regulation of p11 by retinoid signaling and challenges the hypothesis that ubiquitin-mediated proteasomal degradation of p11 represents a universal mechanism of regulation of this protein.

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Acute promyelocytic leukemia (APL), a M3 subtype of acute myeloid leukemia (AML), is associated with poor outcome due to early hemorrhagic death(1). APL is characterized by the chromosomal translocation of the

retinoic acid receptor-alpha gene (*RARα*) on chromosome 17 with the promyelocytic leukemia gene (*PML*) on chromosome 15 that produces the PML/*RARα* fusion oncoprotein. The PML/*RARα* oncoprotein retains features of the *RARα* transcription factor including the ability to bind to other *RARα*-associated proteins and to interact with retinoic acid response elements (RAREs), which are located in the promoter regions of many genes. The presence of PML/*RARα* disrupts endogenous *RARα* functions and regulation of its target genes, consequently exerting a dominant-negative effect that inhibits the *RARα*-mediated differentiation of promyelocytes to granulocytic cell types(2–4). In addition to impaired promyelocyte differentiation, patients with APL present with an abnormal accumulation of promyelocytes and life-threatening hemorrhagic complications resulting from abnormalities in coagulation and fibrinolysis(5).

One of the key processes contributing to the hemorrhagic complications in APL patients is the excessive production of plasmin(5–7), a fibrinolytic factor associated with early deaths in APL patients. Plasmin is an extracellular serine protease generated from its precursor plasminogen through cleavage by plasminogen activators, such as tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA). Plasminogen activation is greatly facilitated by the binding of plasminogen to plasminogen receptors located on the cell surface(8, 9). In many cell types, one of the significant regulators of plasmin generation is the plasminogen receptor, S100A10 (p11). P11 is typically bound to annexin A2 (p36), which forms the annexin A2-p11

heterotetramer complex (AIIIt)(10, 11) (reviewed in (12)). The formation of the complex is necessary to prevent the rapid degradation of p11(13–15). P11 is involved in numerous biological processes including monocyte and macrophage recruitment during the inflammatory response(16, 17), fibrinolysis by endothelial cells(18), and cancer cell metastasis(19–21). We previously showed that induction of PML/*RARα* expression upregulates p11 and p36 protein levels, and plasmin generation *in cellula*(22). Furthermore, depletion of p11 from a patient-derived APL promyelocyte cell line, NB4, resulted in reduced plasmin and fibrinolytic activity, demonstrating the involvement of p11 in the hyperfibrinolysis associated with APL.

The main treatment of APL is all-trans retinoic acid, ATRA, which is a vitamin A metabolite(23) and *RARα* ligand(24). ATRA treatment results in loss of the PML/*RARα* oncoprotein by directly binding to the ligand-binding domain of the *RARα* moiety of PML/*RARα* oncoprotein, which induces protein kinase A (PKA)-phosphorylation, polyubiquitylation and proteasomal degradation of PML/*RARα*(25–27). By destroying the PML/*RARα* oncoprotein, ATRA restores normal *RARα* transcriptional activity thereby allowing promyelocyte differentiation to granulocytes(28).

ATRA-treated NB4 cells also induces the loss of p11 and p36 protein. However, agents that block p36 protein expression have been reported to cause the rapid ubiquitylation and proteasomal degradation of p11(14, 15, 29). Therefore, it is unclear if the ATRA-mediated destruction of p11 is direct via transcriptional regulation of the p11 gene or indirect by affecting the levels of



p36. Although expression of PML/RAR $\alpha$  results in increased expression of p11 and p36 protein, it is unclear if PML/RAR $\alpha$  regulates p11 levels directly or indirectly by its ability to increase p36 levels.

Here, we re-examined the mechanisms regulating the expression of p11 in APL promyelocytes. We present evidence that the ATRA-dependent loss of PML/RAR $\alpha$  in NB4 cells induced transcriptional downregulation and proteasomal degradation of p11 independent of ubiquitylation. Furthermore, transcript and protein levels of p11 in APL promyelocytes are regulated by PML/RAR $\alpha$  oncoprotein expression. Surprisingly, we discovered that ATRA treatment also reduced p11 expression in other cancer cells, hence showing the therapeutic value of ATRA to reduce p11 in APL and other cancer types.

## RESULTS

### *ATRA induces the ubiquitin-independent proteasomal degradation of p11 in APL patient-derived NB4 cells*

Central to our understanding of the regulation of p11, agents that downregulate p36 result in the rapid ubiquitylation and proteasomal degradation of the unpartnered p11(14, 15). Our previous study(30) demonstrated that the ATRA treatment of NB4 cells caused a dramatic loss of p11 and p36 expression. To re-examine if ATRA downregulates p11 protein levels by ubiquitylation and proteasomal degradation, NB4 cells were treated for 48 h with ATRA alone or in combination with the proteasome inhibitor lactacystin (LC), the E1-ubiquitylation enzyme inhibitor PYR-41 or both. Western blot analysis of ATRA-treated NB4 cells revealed a significant

downregulation of p11 and p36 expression of  $3.57\pm 0.04$ -fold ( $P<0.01$ ) and  $2.86\pm 0.11$ -fold ( $P<0.01$ ) respectively (Figure 1A). The presence of PYR-41 did not prevent the ATRA-dependent loss of p11; however, LC reversed the ATRA-dependent loss in p11. NB4 cells treated with ATRA and LC showed increased levels of ubiquitin-conjugated proteins confirming that LC blocked proteasomal degradation. Furthermore, the addition of PYR-41 to cells treated with ATRA and LC prevented the accumulation of ubiquitin-conjugated proteins observed with ATRA and LC treatment, confirming that PYR-41 inhibited protein ubiquitylation. Unexpectedly, higher molecular weight species of p11 were not found in NB4 cells treated with ATRA and LC, suggesting that ubiquitylated p11 was not detectable under these conditions. Our observation that the ATRA-induced loss of p11 and p36 was not prevented by PYR-41 suggested that ubiquitylation may not be necessary for p11 proteasomal degradation. To explore this further, NB4 cells were treated with ATRA alone or in combination with LC, and p11 was immunoprecipitated and analyzed by western blotting for ubiquitin. Interestingly, no ubiquitin-conjugates of p11 were detected when cells were treated with ATRA and LC (Figure 1B and S1), indicating that ATRA did not induce p11 ubiquitylation. Because interaction between p36 and p11 prevents the ubiquitylation of p11(14, 29), we reasoned that if we immunoprecipitated p11 from p36-depleted, then LC-dependent inhibition of the proteasome would result in the accumulation of ubiquitylated, unpartnered p11. As expected, p36-depletion resulted in a

loss of p11 expression, and LC treatment of the p36-depleted cells restored p11 protein levels; however, ubiquitin-conjugated p11 was not detected in LC-treated p36-depleted cells (Figure 1C and S2). The absence of higher molecular weight species of p11 and our inability to detect ubiquitin conjugates of p11 strongly suggested that the loss of p11 protein expression observed with p36-depleted cells was due to ubiquitin-independent proteasomal degradation.

To examine if ATRA could directly regulate p11 independent of PML/RAR $\alpha$ , we chose the MCF-7 cell line since these cells do not express this oncoprotein. MCF-7 cells were treated with ATRA alone or in combination with LC or PYR-41. Although ATRA treatment of MCF-7 cells had no effect on p36 protein levels, p11 was significantly decreased by  $1.82 \pm 0.09$ -fold ( $P < 0.01$ ). In addition, the presence of LC, but not PYR-41, prevented the ATRA-induced loss of p11 expression in MCF-7 cells (Figure 1D). In MCF-7 treated with ATRA and LC, we also did not observe the presence of higher molecular weight species of p11 suggesting the absence of detectable ubiquitylated p11. The effect of ATRA on p11 expression was also assessed in other breast cancer cell lines (MDA-MB-231 and SUM159PT); however, p11 protein levels were not affected (Figure S3). Thus, ATRA treatment can promote p11 proteasomal degradation independently of PML/RAR $\alpha$  and p36, although this phenomena is cell context dependent.

#### ***Forced co-expression of p11 and ubiquitin is required to initiate p11 ubiquitylation***

In order to examine the possibility that other types of proteolytic

regulatory mechanisms might regulate p11 levels, HEK293T cells were transfected with a p11-expressing construct and treated with proteolytic inhibitors. Since p36 and p11 are expressed at low levels in HEK293T cells, we reasoned that the forced expression of p11 in the presence of low intracellular levels of p36 would result in the accumulation of p11 if the appropriate proteolytic regulatory pathway were inhibited. Inhibitors of lysosomal and calpain proteolytic pathways failed to affect p11 levels. P11 accumulated only in the presence of LC, suggesting that the proteasomal regulatory pathway was a key pathway for regulation of p11 levels (Figure 2A). To investigate if p11 was a substrate for ubiquitin-independent degradation by the 20S proteasome, purified p11, p36 or AII $\tau$  proteins were incubated with a purified 20S proteasome preparation. We observed that p11 and p36 were degraded by the 20S proteasome (Figure S4A-C), and this was blocked by LC *in vitro* (Figure S4B-C). Albumin, a negative control, was not proteolyzed under these conditions (Figure S4A). Together, these data support that proteasomal degradation of p11 can be regulated in an ubiquitin-independent manner.

P11 has been shown to be ubiquitylated when co-expressed with ubiquitin in HEK293T cells(14, 29). Hence, p11 was transiently co-expressed with wildtype ubiquitin (ub-WT) or a mutant ubiquitin in which all lysine residues were mutated to arginine (ub-K0) to prevent the formation of polyubiquitin chains that directs proteins for proteasomal degradation(31). Compared to the control group, HEK293T cells overexpressing p11 alone or p11 with ub-WT did not show a

significant increase in p11 expression. However, co-expression of p11 and ub-K0 resulted in  $4.0 \pm 0.45$ -fold ( $P < 0.001$ ) more p11 than control. Furthermore, higher molecular weight forms of p11 were detected only when cells were transfected with both p11 and ub-WT or ub-K0 (Figure 2B), although the latter was more dramatic. The presence of multiple molecular weight species of p11 in cells transfected with ub-K0 suggests the presence of multiple ubiquitin sites on p11. The sites of ubiquitylation on p11 were next identified by mass spectrometry using p11 immunoprecipitated from HEK293T cells co-expressing p11 and ub-K0. We observed that the  $\sim 19.5$  kDa p11 band was ubiquitylated on Lys57 and the  $\sim 28$  kDa p11 band was ubiquitylated on Lys57 and several lysines including 27 or 37 (Figure S5). Next, we performed solvent accessibility analysis of p11. The analysis indicated that Lys57 was 83.5% solvent exposed (Figure S6), making a plausible target for ubiquitylation. In order to validate the sites of ubiquitylation, ubiquitylated p11 was produced by co-transfecting HEK293T cells with vectors expressing ub-KO and p11-WT or p11 mutants. We observed that forced expression of p11-WT or p11-K54R resulted in increased p11 expression when co-expressed with ub-K0 ( $1.37 \pm 0.21$ -fold,  $P < 0.001$  and  $2.79 \pm 0.58$ -fold,  $P < 0.0001$  respectively) and higher molecular weight forms of p11 were apparent. In contrast, co-expressing p11-K57R and ub-K0 did not significantly increase p11 levels and higher molecular weight forms were not observed (Figure 2C). These findings strongly suggest that forced expression of p11 and ubiquitin results in the ubiquitylation of Lys57 of p11.

### ***PML/RAR $\alpha$ is a regulator of p11 expression***

ATRA treatment induces the granulocytic differentiation and degradation of PML/RAR $\alpha$  in NB4 cells, a patient-derived APL promyelocyte cell line. It is not certain whether granulocytic differentiation or the loss of PML/RAR $\alpha$  are responsible for the downregulation of p11 and p36 expression in ATRA-treated NB4 cells. Accordingly, the effect of ATRA on p11 and p36 expression was examined using a NB4 cell line resistant to ATRA-induced differentiation (NB4-MR2)(32). Granulocytic differentiation was assessed using the nitroblue tetrazolium blue (NBT) assay and as expected,  $78.10 \pm 9.3\%$  of ATRA-treated NB4 cells stained positive whereas only  $12.45 \pm 9.32\%$  of ATRA-treated NB4-MR2 cells stained with NBT (Figure S7). Importantly, ATRA treatment (72 h) of both NB4 cell lines produced a loss of PML/RAR $\alpha$ , p11 and p36 expression (Figure 3A), indicating that p11 and p36 expressions are regulated by PML/RAR $\alpha$  and not affected by cell differentiation. The effect of PML/RAR $\alpha$  induction and ATRA treatment was assessed using the U937/PR9 (PR9) cell line that expresses PML/RAR $\alpha$  on a zinc-inducible promoter. PML/RAR $\alpha$  induction in PR9 cells was concomitant with the upregulation of p11 and p36 protein levels (Figure 3B). Since we observed that ATRA induced the loss of p11, p36, and PML/RAR $\alpha$  in NB4 cells, we expected that ATRA would cause a loss of p11 and p36 expression in induced PR9 cells. ATRA treatment reduced p11 and p36 expression in non-induced (by  $2.0 \pm 0.008$ -fold ( $P < 0.0001$ ) and  $1.69 \pm 0.03$ -fold, ( $P < 0.001$ ) respectively) and in induced PR9 cells (by  $1.49 \pm 0.01$ -

fold,  $P < 0.0001$  and  $2.04 \pm 0.001$ -fold,  $P < 0.001$ , respectively) (Figure 3C). ATRA treatment did not affect the expression of p11 or p36 in U937 cells (Figure 3D), the parent cell line of the PR9 cell line(33), suggesting that the ATRA-induced loss of p11 and p36 in the non-induced PR9 cells may be due to 'leaky' expression of PML/RAR $\alpha$  from the zinc-inducible vector. We also observed that p36 levels returned to basal levels in induced PR9-p36 knockdown cells making it difficult to determine if PML/RAR $\alpha$  can increase p11 levels independent of PML/RAR $\alpha$ -induced increases in p36 (data not shown).

#### ***Transcriptional regulation of p11 by ATRA and PML/RAR $\alpha$***

ATRA treatment of NB4 cells decreases the protein levels of p11, but the transcript levels were shown to be unaffected(22) or reduced(34). As shown in figure 4A, the induction of PML/RAR $\alpha$  in PR9 cells resulted in the increase in p11 and p36 transcripts by  $3.65 \pm 0.58$  ( $P < 0.01$ ) and  $3.95 \pm 1.56$ -fold ( $P < 0.01$ ) respectively, suggesting a direct regulation of these genes by PML/RAR $\alpha$ . Consistent with the transcriptional regulation of p11 by PML/RAR $\alpha$ , *in silico* analysis of the  $\pm 10$  kb region from the p11 transcriptional start site showed several potential binding sites for RAR $\alpha$  and RAR $\gamma$ . These sites included canonical retinoic acid response element (RARE) hexameric [RGK TSA] repeats separated by 5-bp direct repeats (DR5)(35) (Table S1, Figure S8). ATRA treatment of NB4 cells significantly reduced p11 and p36 transcript levels by  $10.0 \pm 0.05$ -fold ( $P < 0.0001$ ) and  $6.25 \pm 0.05$ -fold ( $P < 0.0001$ ) respectively compared to the control group (Figure 4B). The ATRA-

stimulated decrease in p11 transcripts was partially reversed by LC and completely reversed for p36 transcripts. LC alone did not cause a significant increase of p11 or p36 transcript levels compared to the control group (Figure S9). This suggested that the reversal of ATRA-mediated decreases in p11 protein by LC were due to both transcriptional and posttranslational regulation. P11 transcript levels were reduced by  $3.21 \pm 0.11$ -fold ( $P < 0.0001$ ) and p36 was increased by  $1.38 \pm 0.23$ -fold ( $P < 0.01$ ) in ATRA-treated MCF-7 cells compared to the control group (Figure 4C), suggesting that ATRA regulates p11 transcription possibly through RAR $\alpha$  activation.

#### ***Overexpression of p36 upregulates p11 protein and transcript levels***

Understanding the mechanisms regulating p11 expression in APL and its treatment by ATRA is complicated by the observation that p11 is only stable after forming a complex with p36, thus implying that agents which decrease p36 protein levels will decrease p11 protein levels. In the absence of p36, p11 has been reported to be unstable and is rapidly ubiquitinated and degraded by the 26S proteasome(13–15). The simplest experimental paradigm for examining the mechanism by which p36 protects p11 is to examine p11 mRNA and protein levels in cells isolated from p36 knockout mice. Hence, cell cultures of macrophages isolated from p36-wildtype (p36<sup>+/+</sup>) or p36-knockout (p36<sup>-/-</sup>) mice were incubated in the presence or absence of LC for 24 h. As shown in figure 5A, total depletion of p36 resulted in a dramatic loss of p11 protein. Surprisingly, p11 protein expression was not rescued by LC treatment, suggesting that the loss of p11 was not due to

proteasomal degradation. Next, we repeated this analysis with HEK293T cells that express low levels of p11 and p36. We reasoned that the forced expression of p36 in the presence of low intracellular levels of p11 and p36 would result in the accumulation of p11 since overexpressed p36 would be available to protect p11 from proteasomal degradation. The overexpression of p36 resulted in a  $1.86 \pm 0.2$ -fold ( $P < 0.001$ ) increase in p11 protein levels (Figure 5B), but did not affect p11 mRNA levels (Figure 5C). The incubation of HEK293T cells with LC failed to affect the levels of p11 protein levels (Figure 5D), suggesting that the proteasome did not regulate p11 levels in these cells and that the increased protein levels of p11 observed in the response of the forced expression of p36 was not due to p36-dependent protection of p11 from proteasomal degradation.

## DISCUSSION

The life-threatening coagulopathy associated with APL is ameliorated primarily by treatment of patients with ATRA. Mechanistically, ATRA blocks the heightened fibrinolytic activity of the leukemic promyelocytes in part by stimulating the degradation of the profibrinolytic protein, p11. The current study was undertaken to elucidate the mechanism(s) by which p11 is regulated in leukemic promyelocytes.

We observed that induction of PML/RAR $\alpha$  resulted in a significant increase in p11 and p36 mRNA. Furthermore, ATRA treatment of NB4 cells resulted in a dramatic decrease in p11 and p36 mRNA. This suggests that PML/RAR $\alpha$  directly regulates p11 transcription. PML/RAR $\alpha$  also indirectly regulates p11 post-translationally by

providing the newly translated p11 protein with its stabilizing binding partner, p36. ATRA indirectly regulates p11 transcription, possibly by blocking PML/RAR $\alpha$ -stimulated p11 transcription and indirectly by blocking p36 transcription resulting in less p36 protein and less stabilized p11 protein. However, a direct effect of ATRA on p11 transcription in NB4 cells is possible as we observed a direct effect of ATRA on p11 transcription in MCF-7 cells, which do not express PML/RAR $\alpha$ . ATRA treatment of the lung epithelial cell line, BEAS-2B, has also been shown to downregulate p11 protein levels(36). Therefore, our data suggests that ATRA regulates p11 protein levels indirectly by both transcriptional and post-translation mechanisms. The simplest explanation for LC-dependent increases in p11 is that LC prevents the ATRA-stimulated proteasomal destruction of PML/RAR $\alpha$ . This would result in increased PML/RAR $\alpha$ -dependent stimulation of p11 and p36 transcription and increased expression of partnered p11 protein.

Our data presents a new model for regulating the p11 protein by ubiquitin-independent proteasomal degradation. This model is supported by our observation that ubiquitin-conjugated p11 is not detected when p11 was immunoprecipitated from ATRA-treated NB4 cells incubated with LC, even though LC caused the accumulation of ubiquitylated proteins in ATRA-treated NB4 cells. Furthermore, when ATRA-treated NB4 cells were incubated with LC, p11 protein levels increased but ubiquitin-conjugated higher molecular weight species of p11 were not detectable. We also observed that the inhibition of protein ubiquitylation by the inhibitor of the E1 ubiquitin-activating enzyme, PYR-41

prevented the LC-dependent accumulation of ubiquitin-conjugated proteins but failed to affect LC-induced increases in p11 protein. Although we cannot rule out the possibility that p11 was de-ubiquitylated during cell lysis and SDS-PAGE analysis, this would be inconsistent with our ability to detect ubiquitylated p11 in 293T cells overexpressing p11 and ubiquitin. Furthermore, EDTA present in the cell lysis buffer removes heavy metal ions necessary for deubiquitylation activity(37). We attempted to use the deubiquitylation inhibitor, N-ethylmaleimide, to prevent any removal of ubiquitin from p11, but this failed to allow detection of any ubiquitin-conjugated p11 species.

The prevailing hypothesis is that in the absence of p36, p11 is unstable and is rapidly ubiquitinated on carboxyl-terminal lysines and directed to the proteasome for degradation. He *et al.*(14), showed by overexpressing a series of carboxyl-terminal terminal mutants of p11 that ubiquitylation was likely to involve Lys92 or Lys94 of the p11 carboxyl-terminal sequence, <sup>89</sup>VHMKQKGKK<sup>97</sup>. However, our data utilized mass spectrometric analysis of overexpressed p11 and identified Lys57 as the primary target of ubiquitylation. Interestingly, Lys57 is a highly conserved lysine that is present in all 16 animal species that have been sequenced and is also surface exposed(38). Furthermore, mapping of endogenous putative ubiquitylation sites in murine tissues by mass spectrometry identified Lys47, Lys54 and Lys57 as the primary ubiquitylated lysines in p11(39), although these sites were not verified by site-directed mutagenesis. Our study shows that mutagenesis of Lys57 prevents the ubiquitylation and

degradation of overexpressed p11 suggesting that this is the key site for ubiquitylation of overexpressed p11.

Proteasomal degradation of proteins can occur by two mechanisms(40): ubiquitin-dependent degradation by the 26S proteasome and ubiquitin-independent degradation by the 20S proteasome, and LC inhibits both of these mechanisms by targeting the catalytic  $\beta$ -subunit of the 20S proteasome core(41). We observed that LC prevented the loss of p11 protein, but inhibition of ubiquitylation did not, suggesting that ubiquitylation of p11 is not required for its degradation by the proteasome. Therefore, we investigated whether p11 was degraded by the proteasome in an ubiquitin-independent manner by incubating purified p11 protein with the 20S proteasome, and we observed that it was rapidly degraded *in vitro*. The presence of intrinsically disordered regions is also a critical feature of proteins degraded in a ubiquitin-independent manner by the 20S proteasome, such as p53 and ornithine decarboxylase (Reviewed in (42, 43)). However, disordered regions are also involved in regulation of ubiquitin-dependent proteasomal degradation. Disordered regions contain short linear motifs, called degrons, that are required for the recognition by E3 ubiquitin-ligases for ubiquitylation, but this is often associated with post-translational modification of the substrate protein(44). Furthermore, the disordered regions also contain lysines targeted for ubiquitylation that are located in close proximity to the E3 ubiquitin-ligases binding motif. Intrinsically disordered regions have been identified in p11 as well as other members of the S100 family of proteins(45), and Lys57 is located in the

helix III region which is an intrinsically disordered region. This suggests that the presence of an intrinsically disordered region around Lys57 of p11 may be critical in regulating both ubiquitin-independent and -dependent proteasomal degradation.

These findings demonstrate that ATRA therapy promotes the downregulation of p11 at the transcriptional and post-translational levels in APL and in some non-APL cells. We present evidence that ATRA induces ubiquitin-independent degradation of p11; a novel mechanism that contradicts the widely accepted paradigm that p11 is regulated by ubiquitin-dependent proteasomal degradation. The forced expression of p11 and ubiquitin empowered the ubiquitylation of p11 at Lys57 and we cannot rule out the possibility that p11 is ubiquitylated at this site under other physiological circumstances. The observation that ATRA affected p11 expression in non-APL cancer cells identifies p11 as a potential RAR $\alpha$  target gene, which may lead to targeting p11 in other cancers using retinoid therapy.

## EXPERIMENTAL PROCEDURES

### *Reagents*

All-trans retinoic acid (ATRA), zinc sulfate (ZnSO<sub>4</sub>), MDL28170, and ammonium chloride (NH<sub>4</sub>Cl) were purchased from Sigma-Aldrich (Oakville, ON, Canada). 4-nitroblue tetrazolium was purchased from Fisher Scientific (Ottawa, ON, Canada). Lactacystin was purchased from Enzo Life Sciences (East Farmingdale, NY, USA). Phorbol 12-myristate 13-acetate (PMA) were purchased from Tocris (Minneapolis, MN, USA). PYR-41 was purchased from BioVision (Milpitas, CA USA). Purified 20S proteasome was

purchased from Boston Biochemical (Boston, MA, USA) or Enzo Life Sciences. Calpain inhibitor IV was purchased from MilliporeSigma (Etobicoke, ON, Canada). Purified 20S proteasome was purchased from Boston Biochemical or Enzo. Ubiquitin expression vectors (pRK5-HA-ubiquitin-wild-type and -K0) were purchased from Addgene.

### *Plasmids*

The cDNA for full-length *p11* was amplified by PCR and ligated into the pcDNA3.1/neomycin (pcDNA-p11) vector (Invitrogen) that constitutively expresses high levels of p11 under the control of the SV40 promoter.

### *Site-directed mutagenesis of p11*

Site-directed mutagenesis of the pcDNA-p11 was performed using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer's instructions, and primers were designed using the QuikChange<sup>®</sup> Primer Design Program. Mutants of p11 were produced using the following primers sets:

P11 Lys54 $\rightarrow$ Arg (K54R)

Fwd – 5'- CCA GGT CCT TCA TTA TTC TGT CCA CAG CCA GAG GG - 3'

Rvs – 5'- CCC TCT GGC TGT GGA CAG AAT AAT GAA GGA CCT GG- 3'

P11 Lys57 $\rightarrow$ Arg (K57R)

Fwd – 5'- TGG TCC AGG TCC CTC ATT ATT TTG TCC ACA GCC AGA- 3'

Rvs – 5'- TCT GGC TGT GGA CAA AAT AAT GAG GGA CCT GGA CCA- 3'

### *Cell culture*

NB4-MR2 and U937/PR9 (PR9) cells, which express PML/RAR $\alpha$  under the control of the zinc-inducible promoter cells, (kindly provided by Dr. Wilson Miller Jr., McGill University, Montreal, QC) and NB4 and U937 cells (DSMZ) were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin. MCF-7, MDA-MB-231, SUM159PT cells (kindly provided by Dr. Paola Marcato, Department of Pathology, Dalhousie University, Halifax, NS) and HEK293T cells (ATCC) were maintained in DMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin. Non-adherent cells were maintained with the cell density kept at  $< 1 \times 10^6$  cells/mL.

#### *Mice*

The p36-deficient mice (p36<sup>-/-</sup>) and their wildtype counterparts (p36<sup>+/+</sup>) are on a 129SV x C57BL/6 background, and were a generous gift from Dr. K. Hajjar (Cornell University, Ithaca, NY)(46). Experimental mice were typically 6-8 weeks of age and comprised both sexes. All animal experiments were performed in accordance with protocols approved by the University Committee on Laboratory Animals at Dalhousie University.

#### *Peritoneal Macrophage Isolation*

Peritoneal macrophages were isolated according to Holloway *et al.*(47)

#### *Treatment with ATRA*

NB4 and PR9 cells were seeded at a density of  $0.3 \times 10^6$  cells/mL and were exposed to ATRA after 24 h. MCF-7 cells were seeded to 6-well plates using  $0.25-0.35 \times 10^6$  cells/well and were exposed to ATRA after 24 h. Stock

solutions of ATRA were diluted in DMSO (Sigma-Aldrich) and added to medium at a final concentration of 1  $\mu$ M. Cells were grown in medium for the indicated times with ATRA or vehicle control added to media daily. NB4 cell density was maintained at  $< 1 \times 10^6$  cells/mL throughout the experiment.

#### *Zinc-Induction of PML/RAR $\alpha$ in PR9 cells*

PR9 cells (seeded at  $0.3-0.5 \times 10^6$  cells/mL) were treated with 100  $\mu$ M ZnSO<sub>4</sub> daily. Cell density was maintained at  $< 1 \times 10^6$  cells/mL throughout the experiment.

#### *Transient transfection*

HEK293T cells were seeded in 6-well plates ( $2.0 \times 10^5$  cells/well) and transfected the following day using the Lipofectamine2000<sup>TM</sup> transfection reagent (Invitrogen) in serum-free OPTI-MEM medium (Invitrogen), according to manufacturer's instructions.

#### *Immunoblot analysis and immunostaining*

Cells were lysed with RIPA lysis buffer [1% Triton-X100, 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, and proteinases and phosphatase inhibitor cocktails (1X final concentration; Thermo Scientific), pH 7.4]. Total protein of cell lysates (40  $\mu$ g) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using 10-20% gels (or 5% gels for PML/RAR $\alpha$  immunoblot) and electrotransferred onto nitrocellulose membranes. The following antibodies were used for immunoblotting: p11, p36 (BD Biosciences),  $\beta$ -actin (Sigma), RAR $\alpha$  (Santa Cruz, C-20), ubiquitin (Cell Signaling) and the secondary antibodies IRdye-800 goat anti-mouse antibody



(LI-COR Biosciences) and IRdye-680 goat anti-rabbit antibody (Thermo Fisher). Antibody complexes were viewed on the Odyssey IR imaging system (LI-COR Biosciences). Protein expression was quantified using Image J software.

#### *Immunoprecipitation*

For immunoprecipitation, cell were lysed in cell lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% NP-40, 1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM EDTA, and complete EDTA-free protease and inhibitor cocktail (Thermo)] and 200 µg of precleared cell lysates were incubated with antibodies for mouse IgG1(R&D) or p11 (BD) for 1 h at 4°C. Afterwards, the lysates were incubated using protein G-agarose or protein A agarose (Santa Cruz) beads for 1 h at 4 °C to collect immune complexes (antibody bound to the target protein). The beads were washed four times in cell lysis buffer, and the immune complexes were eluted from the beads by addition of 40 µL 2X SDS sample buffer and incubation at 50°C for 10 min. The supernatants of the eluted samples were then used for western blot analysis.

#### *In vitro Proteasomal Degradation Assay*

The ability of the 20S proteasome to degraded purified recombinant proteins was assessed using the 20S proteasome assay kit (Boston Biochemical) according to manufacturer's protocols. Briefly, 'reaction buffer' was diluted to 1X and the 3% SDS 'proteasome activation' solution was added to the buffer at a final concentration of 0.03%. Samples were prepared in this buffer without or with 1 µg of purified 20S proteasome alone or in combination with 250 µM

lactacystin (reconstituted in deionized water [dH<sub>2</sub>O]). Next, 1 µg of purified recombinant human proteins ( p11, p36, or AII<sub>t</sub> proteins) were added to the mixture at a final volume of 20 µL and incubated for 1 h at 37°C. The reaction was stopped by the addition of 20 µL of 2X sample loading buffer, and then boiled in water for 5 minutes. The protein lysate were resolved by SDS-PAGE and analyzed by immunoblot analysis or coomassie blue staining for overnight.

#### *Quantitative PCR (qPCR) analysis*

RIBOzol RNA extraction reagent (Amresco) was used to extract RNA from cells according to manufacturer's instruction. Briefly, cells were lysed using 1 mL of RIBOzol and transferred to an Eppendorf tube. Next, 200 µl of chloroform was added the mixture, shaken vigorously, and incubated at room temperature for 5 min. The mixture was centrifuged at 12,000 x g for 10 min at 4 °C and then the aqueous phase was collected and used to purify total RNA using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's protocols. The cDNA was synthesized from total RNA (1 µg) using the QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's protocols. Starting with 25 ng of cDNA, the reaction as carried out using the SSO Advanced Universal SYBR Green Supermix (BioRad Laboratories) and the CFX96 Real-Time PCR Detection System (Bio-Rad) to amplify the genes of interest using the following primer sets (final concentration of 0.5 µM; IDT):

Human p36: NCBI Reference Sequence: NM\_001002858.2

Forward:5'-  
CAAGACCAAAGGTGTGGATG-3'  
Reverse: 5'-  
CAGTGCTGATGCAAGTTCCT-3'

less than 0.05. All data are expressed as mean  $\pm$  S.D.

Human p11: NCBI Reference Sequence:  
NM\_002966.2  
Forward:5'-  
GGACCAGTGTAGAGATGGCA-3'  
Reverse: 5'-  
TTATCAGGGAGGAGCGAACT-3'

Human Gapdh: NCBI Reference  
Sequence: NM\_002046.5  
Forward:5'-  
TCAAGAAGGTGGTGAAGCAG-3'  
Reverse: 5'-  
CGCTGTTGAAGTCAGAGGAG-3'

Human  $\beta$ -actin: NCBI Reference  
Sequence: NM\_001101.3  
Forward:5'-  
ACGTTGCTATCCAGGCTGTG-3'  
Reverse: 5'-  
GAGGGCATACCCCTCGTAGA-3'

Human Hprt1: NCBI Reference  
Sequence: NM\_000194.2  
Forward:5'-  
TTGCTTTCCTTGGTCAGGCA-3'  
Reverse: 5'-  
ATCCAACACTTCGTGGGGTC-3'

Fold change values were calculated using the  $\Delta\Delta C_t$  method(48) and normalized to  $\beta$ -Actin, GAPDH, and H RTP1 expression. An unpaired *t*-test was used to calculate statistical significance.

#### *Statistical analysis*

Statistical significance was determined by Student *t*-test or one-way ANOVA with Tukey multiple comparisons. Results were considered as significant if two-tailed P values were

## **ACKNOWLEDGEMENTS**

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## **AUTHORSHIP**

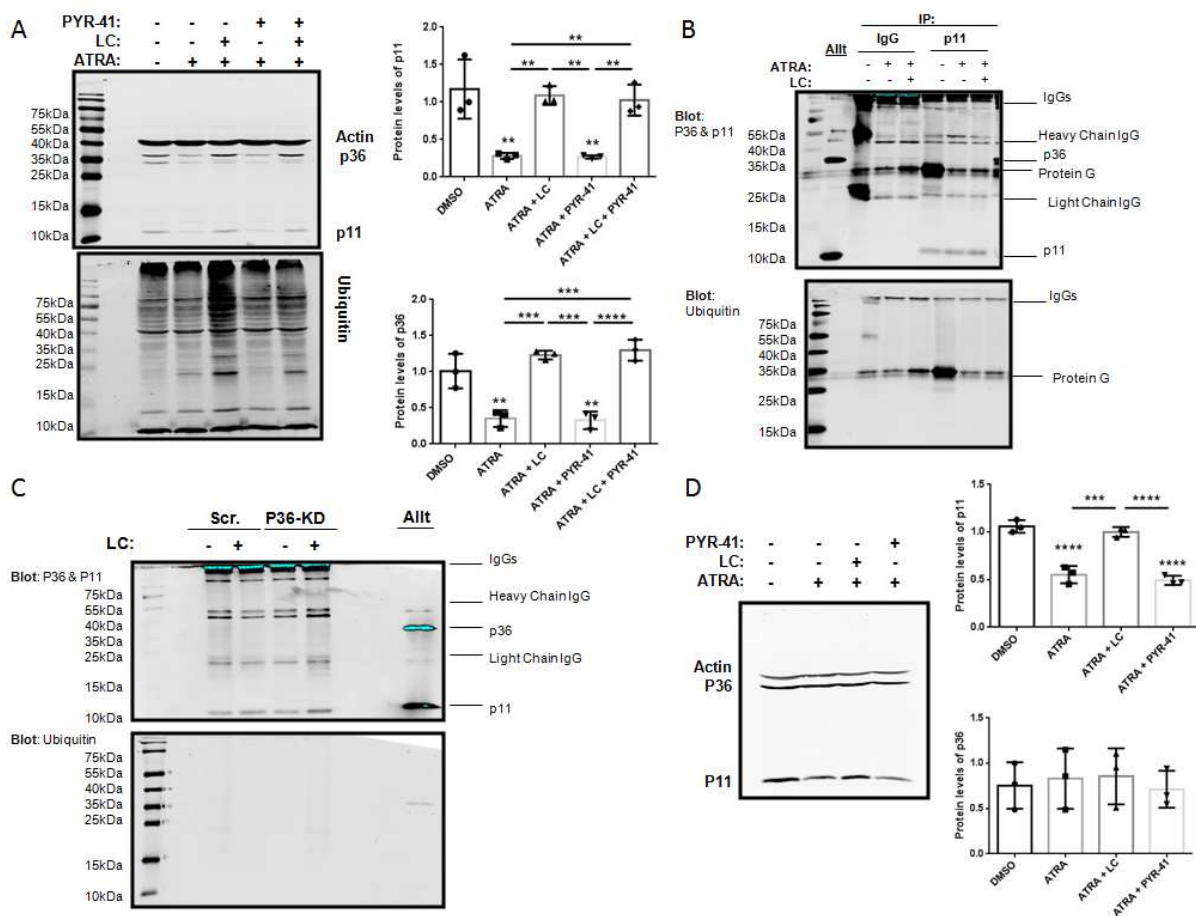
Contribution: R.W.H designed and performed research, analyzed data, and wrote manuscript; M.T and A.C performed research and analyzed data; M.R. performed research; P.M and P.M. designed research and analyzed data; D.M.W designed research, analyzed data, and wrote manuscript.

## **CONFLICT-OF-INTEREST DISCLOSURE**

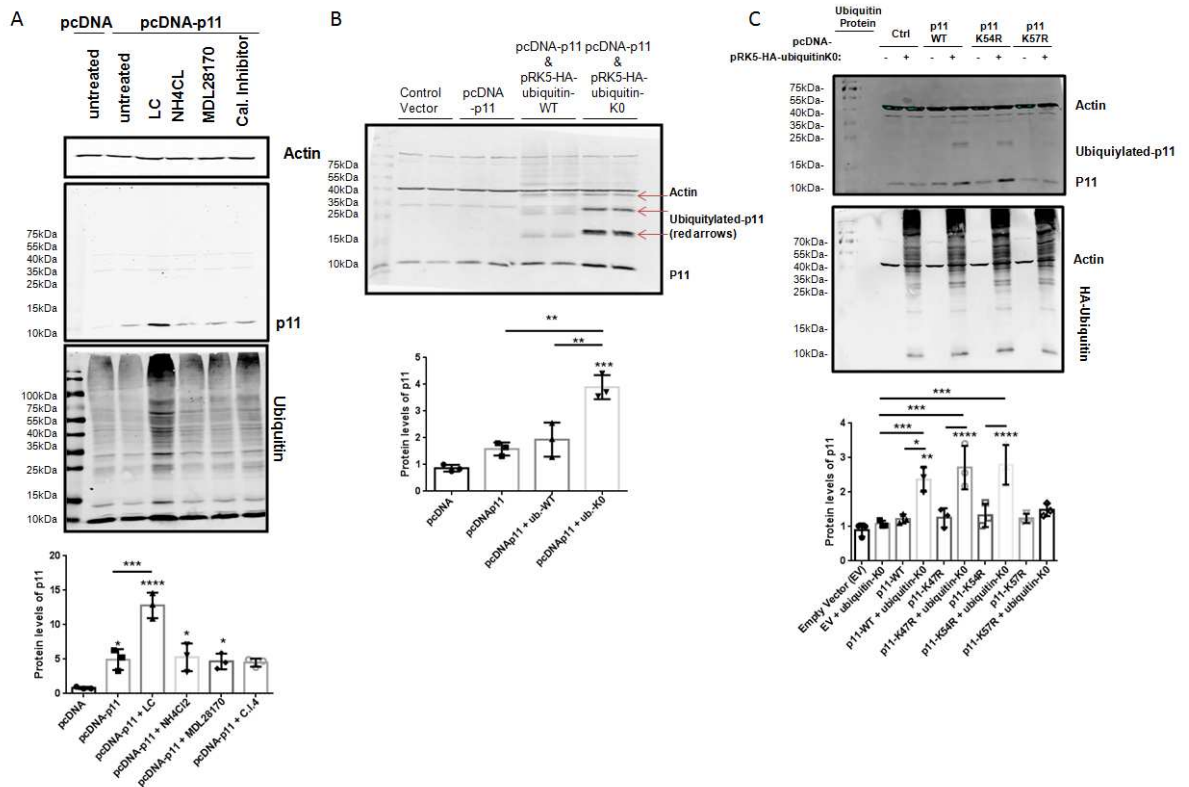
The authors declare no competing financial interests.

## **CORRESPONDENCE**

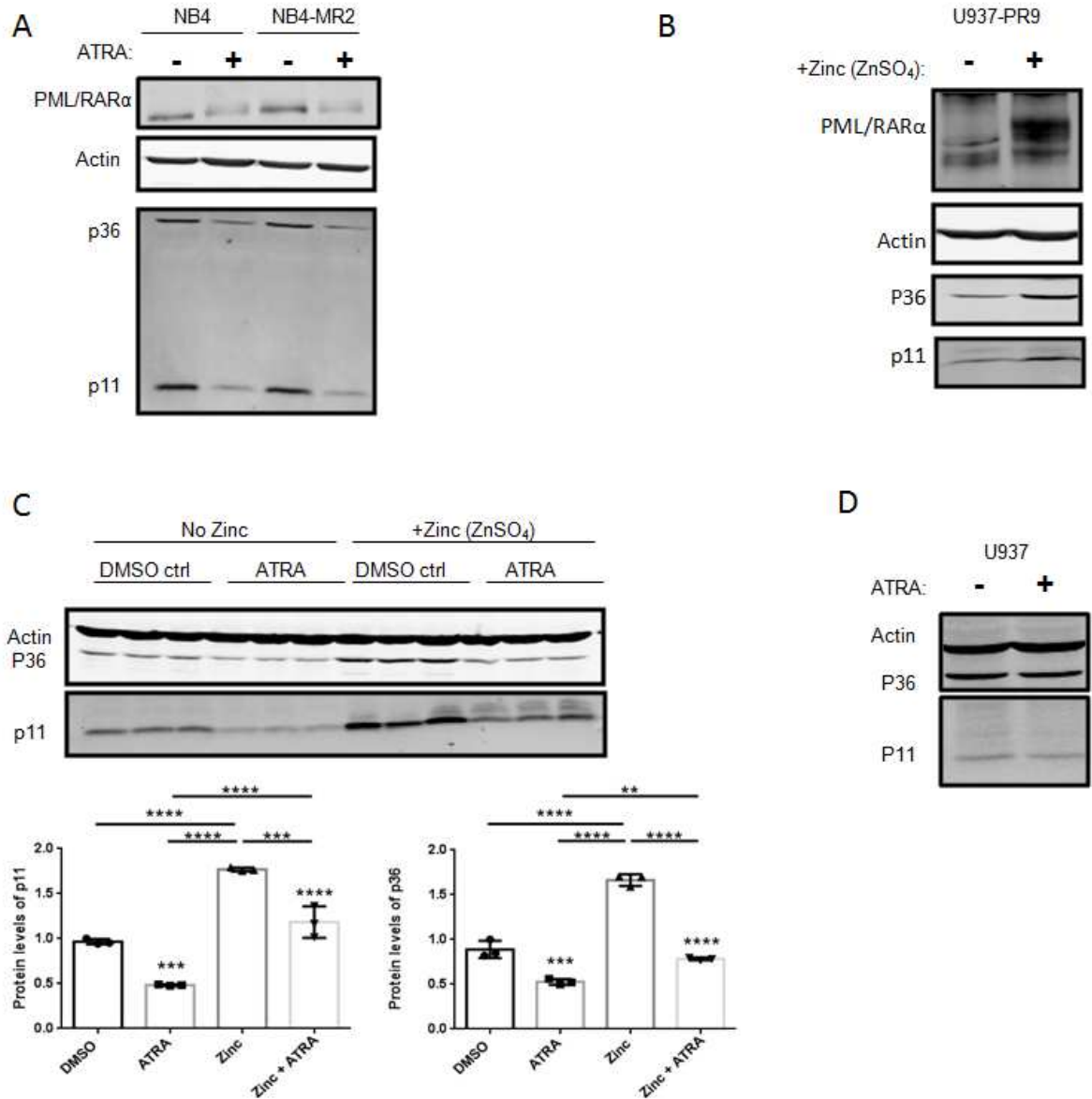
David M. Waisman, Department of Biochemistry & Molecular Biology, Dalhousie University, Halifax, Nova Scotia, B3H 4R2 P.O. Box 15000 Canada; e-mail: [david.waisman@dal.ca](mailto:david.waisman@dal.ca)



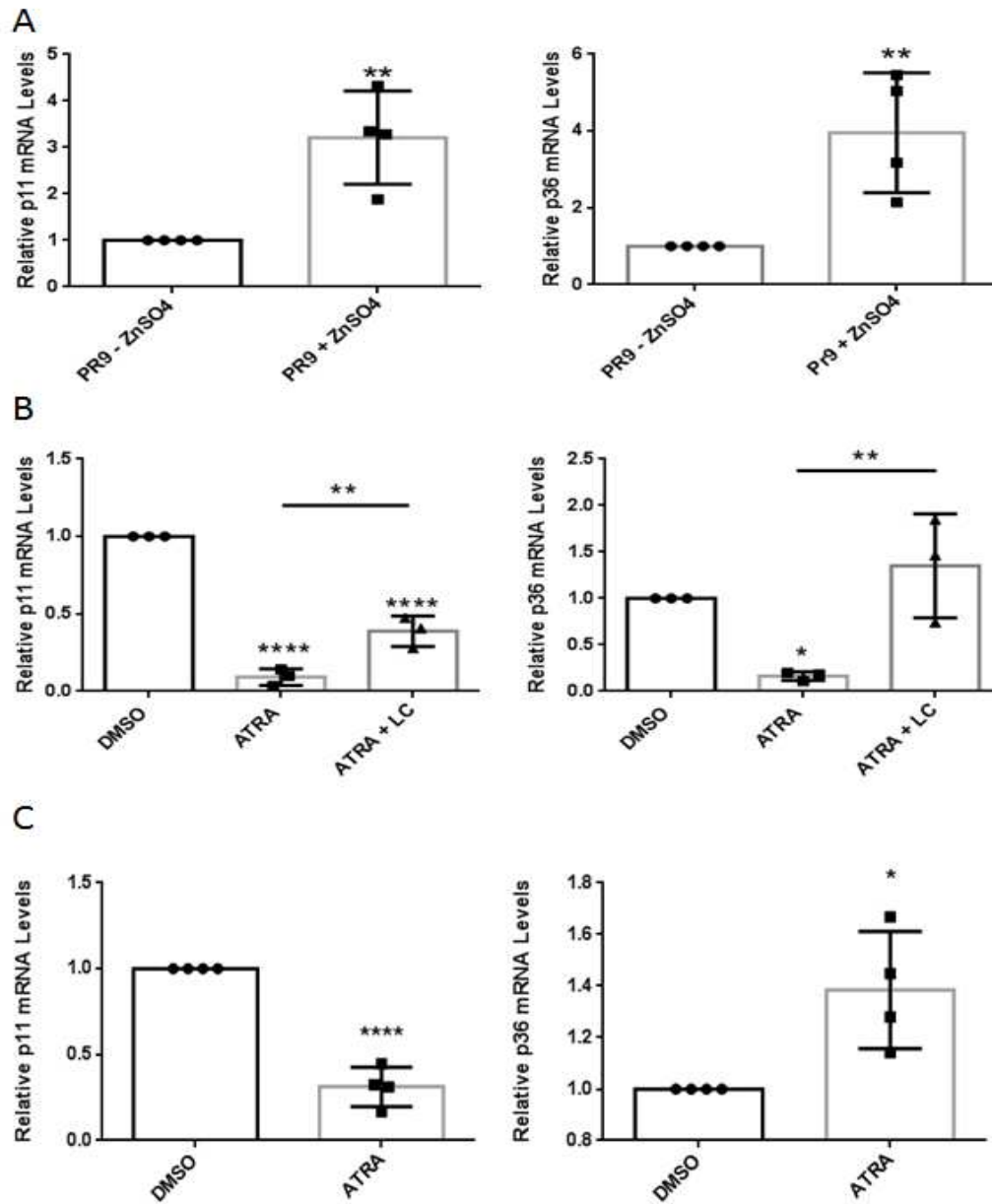
**Figure 1: ATRA induces the ubiquitin-independent proteasomal degradation of p11 in APL cell line, NB4, and MCF-7 breast cancer cells.** (A) Immunoblot analysis of NB4 cells treated for 48 h with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M lactacystin (LC) or 2  $\mu$ M PYR-41. (B) Immunoprecipitation of p11 or IgG1 isotype control in NB4 cells treated for 24 h with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M LC. Purified Allt (0.25  $\mu$ g) was used as a control. (C) Immunoprecipitation of p11 in PR9 cells treated for 48 h with 2  $\mu$ M LC. Purified Allt (0.25  $\mu$ g) was used as a control. (D) Immunoblot analysis of MCF-7 cells treated for 48 h with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M lactacystin (LC) or 2  $\mu$ M PYR-41. Cell lysates were prepared and the level of the indicated proteins were examined by immunoblot analysis with  $\beta$ -actin used as a loading control. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$  are considered statistically significant.



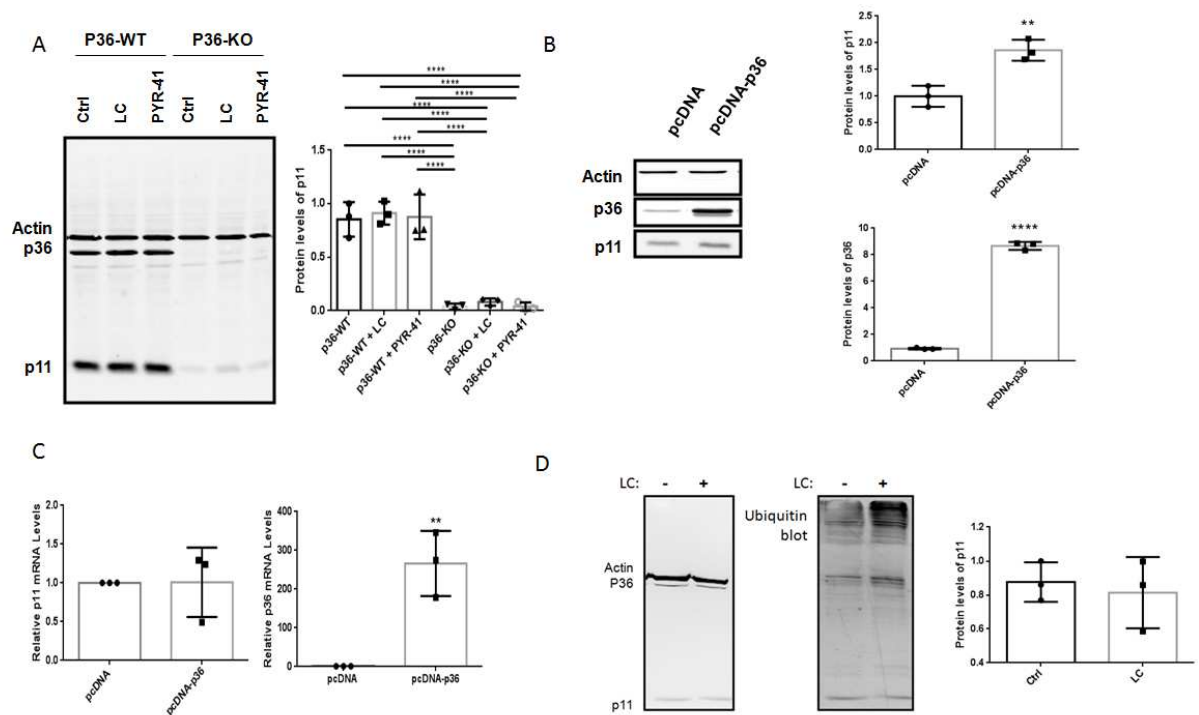
**Figure 2: Lysine 57 is the site of ubiquitylated of p11, but may not be involved in proteasomal degradation.** (A) Immunoblot analysis of HEK293T cells transiently transfected using pcDNA3.1-empty vector or pcDNA3.1-p11 vector alone or treated for 18 h using 3  $\mu$ M lactacystin (LC), 1 mM NH<sub>4</sub>Cl, 1  $\mu$ M MDL28170, or 1  $\mu$ M calpain inhibitor IV (C.I.4). (B) Immunoblot analysis of HEK293T cells transiently transfected using pcDNA3.1-empty vector or pcDNA3.1-p11 vector alone or in combination with pRK5-HA-ubiquitin wildtype (ub-WT) or mutant, lysine-less ubiquitin (ub-K0). (C) Immunoblot analysis of HEK293T cells transiently expressing p11-wild type (p11WT) or p11 mutants (with Lys54 or Lys57 changed to arginine: p11K54R and p11K57R, respectively) alone or in combination with pRK5-HA-ub-K0. Cell lysates were prepared and the levels of the indicated proteins were examined by immunoblot analysis with  $\beta$ -actin used as a loading control. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, and \*\*\*\*P < 0.0001 are considered statistically significant.



**Figure 3: ATRA downregulates p11 and p36 expression through the loss of PML-RAR $\alpha$  expression in APL cells.** Cell lysates were prepared from (A) NB4 or NB4-MR2 were treated without or with 1  $\mu$ M ATRA for 72 h, (B) PR9 cell treated with or without 100  $\mu$ M zinc sulfate (ZnSO<sub>4</sub>) for 48 h, (C) ATRA-treated (1  $\mu$ M for 48 h) PR9 ( $\pm$ 100  $\mu$ M ZnSO<sub>4</sub>) for 48 h and (D) ATRA-treated (1  $\mu$ M for 48 h) U937 cells. Cell lysates were prepared and the levels of the indicated proteins were examined by immunoblot analysis with  $\beta$ -actin used as a loading control. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using one-way ANOVA (with Tukey multiple comparisons), where \*\*P < 0.01, \*\*\*P < 0.001 and \*\*\*\*P < 0.0001 are considered statistically significant.



**Figure 4: Transcript levels of p11 and p36 are regulated by PML-RAR $\alpha$  and ATRA treatment.** Total RNA extracted from (A) PR9 cells treated (48 h) without or with 100  $\mu$ M ZnSO<sub>4</sub>, (B) NB4 cells treated (48 h) without or with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M LC, and (C) ATRA-treated (48 h) MCF-7 cells were used for cDNA synthesis. The relative expression of p11 and p36 mRNA levels was determined from cDNA (25 ng) by qPCR analysis and normalized to GAPDH,  $\beta$ -actin and HPRT1. Data is expressed as the mean  $\pm$  S.D. of three (B) or four (A, C) independent experiments. Statistical significance was determined using (A, C) the Student t-test for unpaired observations or (B) one-way ANOVA (with Tukey multiple comparisons), where \*P < 0.05, \*\*P < 0.01, and \*\*\*\*P < 0.0001 are considered statistically significance.



**Figure 5: P36 upregulates p11 protein without affecting transcript levels.**

Immunoblot analysis of (A) peritoneal macrophages isolated from  $p36^{+/+}$  and  $p36^{-/-}$  mice treated for 24 h using 3  $\mu$ M LC or 10  $\mu$ M PYR-41 and (B) HEK293T cells transiently transfected using pcDNA3.1-empty vector or pcDNA3.1-p36 vector. Cell lysates were prepared and the levels of the indicated proteins were examined by immunoblot analysis with  $\beta$ -actin used as a loading control. Total RNA extracted from (C) HEK293T cells transiently transfected using pcDNA3.1-empty vector or pcDNA3.1-p36 vector were used for cDNA synthesis. The relative expression of p11 and p36 mRNA levels was determined from cDNA (25 ng) by qPCR analysis and normalized to GAPDH,  $\beta$ -actin and HPRT1. (D) Immunoblot analysis of HEK293T cells treated for 16 h using 2.5  $\mu$ M lactacystin (LC). Cell lysates were prepared and the levels of the indicated proteins were examined by immunoblot analysis with  $\beta$ -actin used as a loading control. Data is expressed as the mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using (B, C) the Student t-test for unpaired observations or (A, D) one-way ANOVA (with Tukey multiple comparisons), where \*\* $P < 0.01$  and \*\*\*\* $P < 0.0001$  are considered statistically significant.



## **APPENDIX B: SUPPLEMENTAL DATA**

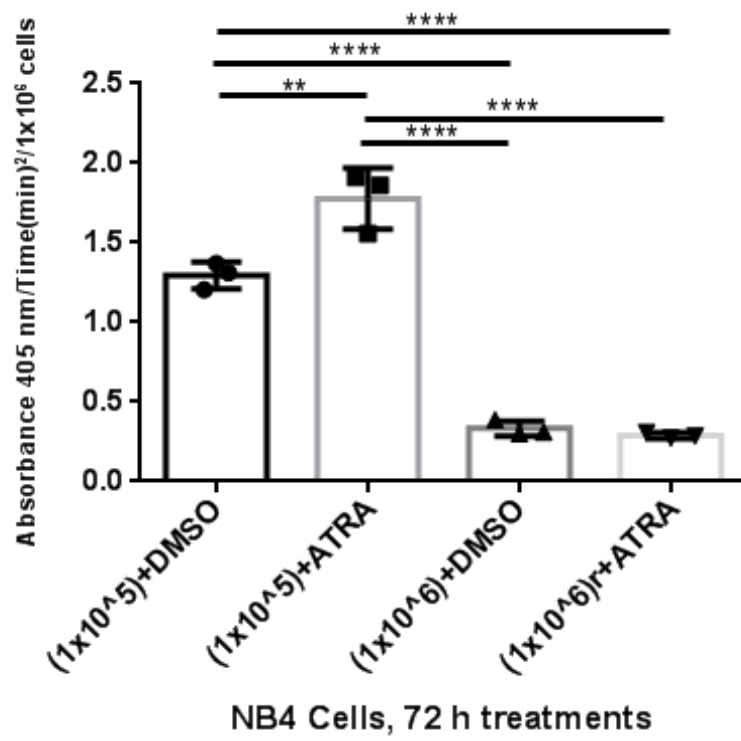
### Supplemental Methods

#### **Cell surface biotinylation**

NB4 cells were centrifuged for 5 min at 300 x g and were washed 3 times in 5 mL of ice cold 1X DPBS. Sulfo-NHS-biotin (Thermo Scientific; 5 mL of a 1 mg/ml solution, ice cold, made in 1X DPBS) was added to the cells, and incubated for 30 min at room temperature. The biotin solution was removed, and cells were washed 3 times in 5 mL of 100 mM glycine (ice cold, made in 1X DPBS). Cells lysates were prepared and 500 µg of total protein (500 µg) was obtained from of each sample. Dynabead mixture (50 µL) was added to each cell lysate and incubated at room temperature for 30 min 1.5 h. Samples were then placed on the dynabead magnet for 2 min, and dynabeads were washed 5 times in 1 mL of 1XDPBS (+0.1% BSA). The dynabeads were resuspended in 25 µL of 2X sample loading buffer and boiled for 5 min.

**Figure S1: Difference in the rate of plasmin generation due to cell numbers seeded into 96-well plates.**

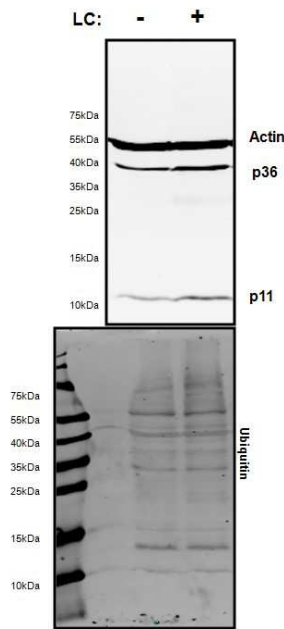
NB4 cells treated with ATRA for 72 h were incubated with plasminogen (0.5  $\mu$ M), seeded to 96-well plates at  $1 \times 10^6$  and  $1 \times 10^5$  cells/well, and the rate of plasmin generation was measured at 405 nm over 4 h and data is expressed as 405 nm/min<sup>2</sup>/1 x 10<sup>6</sup> cells. Data is expressed as mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using the Student t-test for unpaired observations, where \*\*P < 0.01 and \*\*\*\*P < 0.0001 are considered statistically significant. The plasmin generation assays were performed by Dr. Alamelu (Dharini) Bharadwaj and Ryan Holloway.



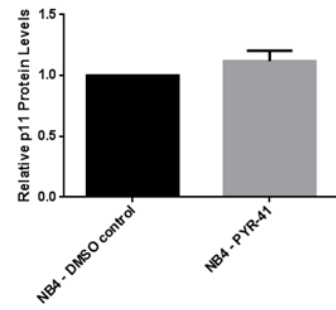
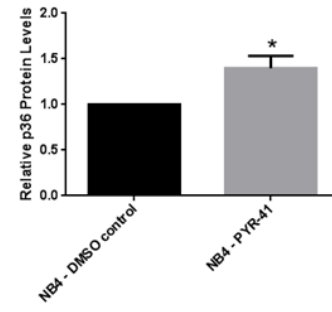
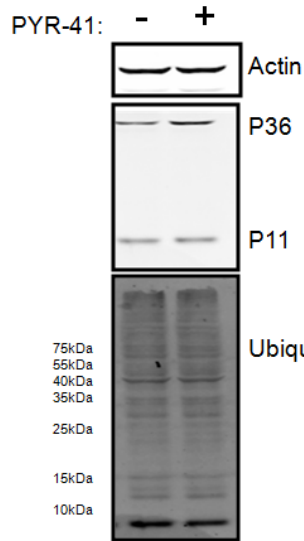
**Figure S2: LC treatment upregulates p11 and p36 protein levels in NB4 cells, but PYR-41 only up-regulates p36.**

Cell lysates were prepared from NB4 with (A) 2  $\mu$ M LC or (B) 2  $\mu$ M PYR-41 for 72 h. The level of p11 and p36 was examined by western blot analysis with  $\beta$ -actin used as a loading control. Data is expressed as mean  $\pm$  S.D. of three independent experiments. Statistical significance was determined using the Student t-test for unpaired observations, where \*P < 0.05 is considered statistically significant.

A

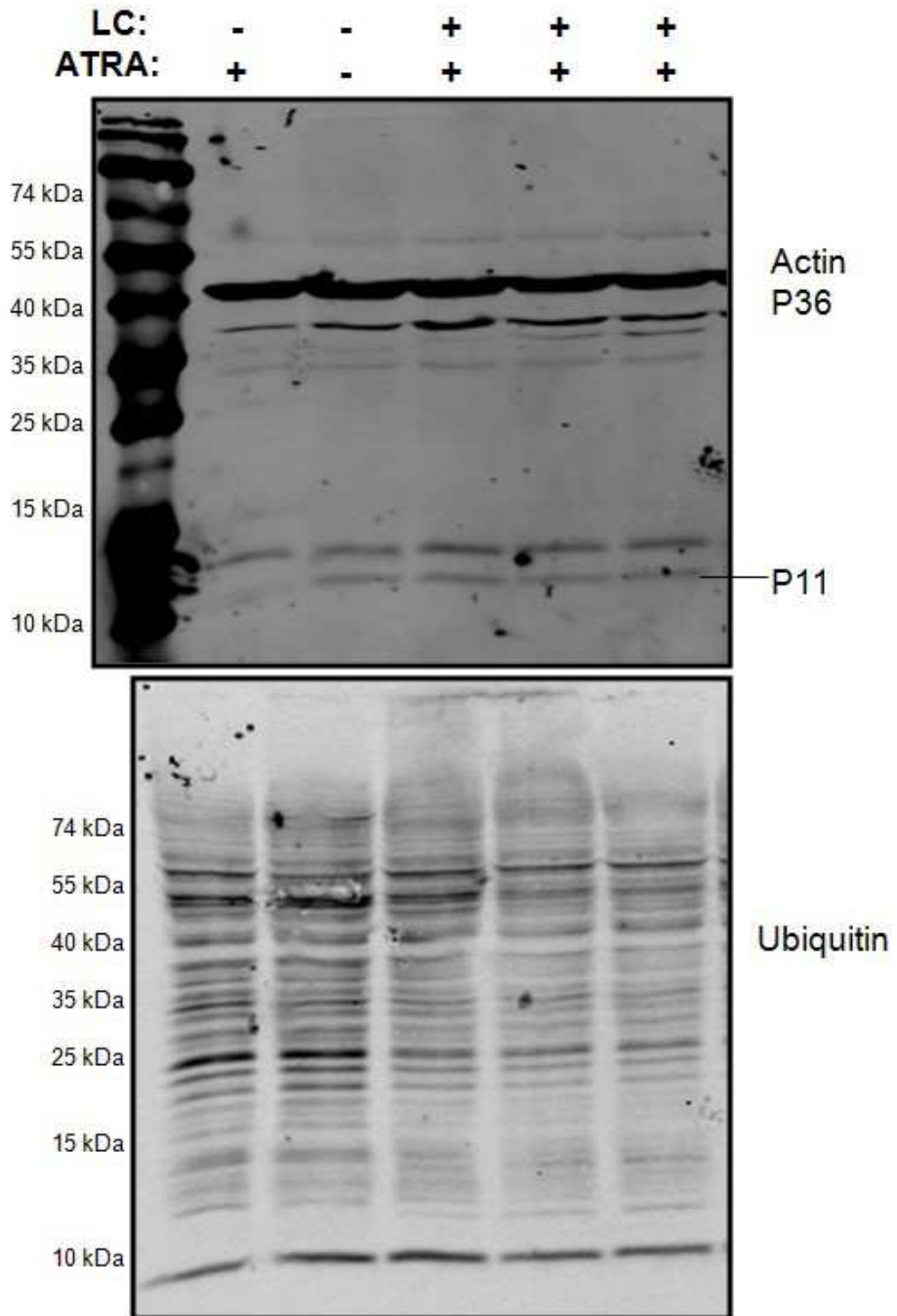


B



**Figure S3: Ubiquitylated p11 does not appear in ATRA-and LC-treated NB4 cells in the presence of the deubiquitinase inhibitor, *N-Ethylmaleimide* (NEM).**

NB4 cells were treated for 48 h with 1  $\mu$ M ATRA alone or in combination with 2  $\mu$ M lactacystin. Cell lysates were prepared with lysis buffer containing 1 mM of *N-Ethylmaleimide* (NEM), and the level of the indicated proteins were examined by western blot analysis with  $\beta$ -actin used as a loading control. Data is expressed as three independent experiments.



**Figure S4: Cell surface levels of p11 and p36 on A549 cells.**

A549 cells were incubated with Sulfo-NHS-SS-biotin and cell lysates were prepared. The biotinylated (cell surface) proteins were collected using Dynabeads and analyzed by western blot analysis.



