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# "HSP90 INHIBITION MEDIATED AXL RECEPTOR TYROSINE KINASE DOWNREGULATION - MOLECULAR MECHANISM AND THERAPEUTIC IMPLICATION"

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# “HSP90 INHIBITION MEDIATED AXL RECEPTOR TYROSINE KINASE DOWNREGULATION - MOLECULAR MECHANISM AND THERAPEUTIC IMPLICATION”

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# LIST OF PUBLICATIONS

This dissertation is based upon the following publications. The first represents the core of the thesis while the second one is attached at the end of the thesis.

1. **Gnana Prakasam Krishnamoorthy**, Teresa Guida, Luigi Alfano, Elvira Avilla, Massimo Santoro, Francesca Carlomagno and Rosa Marina Melillo. Molecular mechanism of 17-Allylamino-17-demethoxygeldanamycin (17-AAG) induced AXL degradation. (Manuscript Submitted).

2. Avilla E, Guarino V, Visciano C, Liotti F, Svelto M, **Krishnamoorthy G**, Franco R, Melillo RM. Activation of TYRO3/AXL tyrosine kinase receptors in thyroid cancer. Cancer Res. 2011;71,1792-1804.

## ABSTRACT

The Tyrosine kinase receptor AXL belongs to the TAM (Tyro3, AXL and Mer) family, whose members are characterized by a highly conserved kinase domain and adhesion molecule-like domains in the extracellular region. There are several reports showing AXL overexpression in cancer and its transforming potential, depicting its ability to drive tumor formation, progression and resistance to different anti-cancer agents; hence, AXL receptor is a clinically validated cancer target. Heat shock protein 90 (HSP90) is a critical player in regulating the maturation, stability, and activity of its client proteins. HSP90 inhibition causes depletion of multiple oncogenic client proteins, leading to blockade of many key cancer causing pathways. HSP90 inhibitors that derived from geldanamycin, such as 17-AAG, have emerged as promising therapeutic agents for cancer treatment and few of them have entered clinical trials.

In this study we show that AXL is a novel client of HSP90, as the two structurally unrelated HSP90 inhibitors Radicicol or 17-AAG, induced a time- and dose-dependent downregulation of endogenous or ectopically expressed AXL protein. Using biotin and $^{35}$S-methionine labelling of the biogenesis/trafficking receptor upon 17-AAG treatment we showed that 17-AAG induced depletion of AXL on the plasma membrane, by mediating degradation of fully glycosylated AXL receptor from internal organelles and therefore restricting its transport to the cell surface. We demonstrated that 17-AAG induced AXL polyubiquitination and subsequent degradation via proteasome. HSP90 functions in the context of a multi-protein chaperone complex. By co-immunoprecipitation experiments, interactions between AXL and the components of chaperone complex like HSP90 itself, HSP70, and Ubiquitin E3 ligase CHIP (Carboxy terminus of Hsc70 Interacting Protein) were shown to be modulated by 17-AAG treatment. By contrast to wt CHIP, the functionally inactive CHIP mutant, CHIP K30A failed to accumulate AXL polyubiquitinated species even in the presence of 17-AAG, suggesting the involvement of endogenous CHIP in 17-AAG induced AXL polyubiquitination. We could also show that, it is the intracellular domain (ICD) of AXL containing the TK domain that is crucial to acquire 17-AAG sensitivity, as the AXL ICD-deleted mutant is insensitive to 17-AAG mediated degradation. However, AXL kinase activity is not required for 17-AAG mediated AXL loss, since inhibition of kinase activity by Bosutinib did not affect AXL sensitivity to 17-AAG. Similarly, AXL kinase dead mutant was as sensitive as the wt receptor to 17-AAG induced degradation. Finally, we also

demonstrated that 17-AAG induced AXL downregulation could interfere with AXL mediated biological activity, measured as activation of transcription from the AP1 promoter, suggesting a possible therapeutic approach to counteract AXL driven oncogenic activity or drug resistance. Our data convincingly clarify the molecular basis of AXL downregulation by HSP90 inhibitor 17-AAG and suggest that HSP90 inhibition in anti-cancer therapy can exert its effect through inhibition of multiple kinases, including AXL itself.

# 1. BACKGROUND

## 1.1 Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are transmembrane proteins that act as growth factor receptors and transduce signals from the extracellular environment to the cytoplasm and nucleus. Thereby, they regulate normal cellular processes such as survival, growth, proliferation, differentiation, adhesion, and motility. There are 58 RTKs in the human genome, which have been classified into 20 families based on sequence similarities within the kinase domain and structural similarities within their extracellular regions (Robinson et al. 2000). Activation of the kinase domains and further downstream signaling is enabled by receptor oligomerization, in most cases dimerization, as a consequence of ligand binding that brings two receptor-chains together, or through ligand-independent oligomerization processes (Schlessinger 2000, Ullrich and Schlessinger 1990). The kinase domain, when activated, transfers a phosphate group from adenosine-5'-triphosphate (ATP) to selected tyrosine residues. The phosphorylation occurs on the receptor at tyrosine residues in the activation loop, which stabilizes the active form of the receptor, but also on other tyrosine residues that, when phosphorylated, serve as docking sites for several downstream intracellular signaling proteins, that harboring Src Homology 2 (SH2) or Phosphotyrosine binding (PTB) structural motifs, SH2 and PTB-containing proteins, interact with the activated receptor (Pawson 2004) and transduce the signalling. The binding specificity of these intracellular signaling molecules is determined by the amino acid sequences surrounding each phosphotyrosine residue (Pawson 2004). Abnormal overexpression or aberrant activation of RTKs has been associated to a variety of human cancers.

### 1.1.1 TAM Family of Receptor Tyrosine Kinases

#### 1.1.1.1 *TAM Receptors*

The TAM family of RTKs includes three transmembrane receptors Tyro3, AXL and Mer that share unique adhesion-molecule-like extracellular region, characterized by two aminoterminal immunoglobulin (Ig)-like domain, and two fibronectin-type III (FNIII) repeats, as well as a conserved carboxy-terminal KW(I/L)A(I/L)ES signature motif within their highly conserved intracellular kinase domain (O'Bryan et al. 1991) (Fig.1). TAM

members share the vitamine K-dependent ligands Gas6 and Protein S (Linger et al. 2008). Gas6 was first identified as a ligand for AXL in 1995 (Stitt et al. 1995; Varnum et al. 1995). The related vitamin K-dependent anticoagulation factor, Protein S, was described as a ligand for Tyro-3 (Stitt et al. 1995). Although numerous subsequent studies confirmed that Gas6 binds to and activates all three members of the TAM receptor family, the validity of Protein S as a ligand for any of the TAM receptors became subject to extensive debate (Godowski et al. 1995; Mark et al. 1996; Nagata et al. 1996) and was established that AXL binds only to Gas6 and its affinity is also higher than its binding to Tyro3 or Mer. Among the TAM family receptors, AXL and Tyro3 have most similar genomic structure sharing the same number and size of exons (Linger et al. 2008). In contrast AXL and Mer have the most similar tyrosine kinase domain amino acid sequence (Robinson et al. 2000).

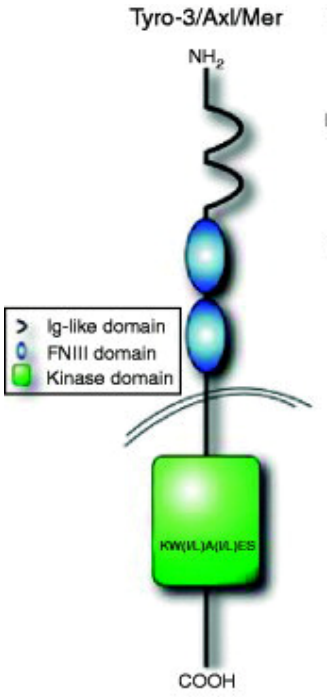


**Figure.1**. Illustrative picture of TAM Family RTKs (adapted from Linger et al. 2008).

### 1.1.1.2 *Expression Profile*

TAM receptor mRNA has been observed in embryonic tissues (Crosier et al., 1996; Faust et al. 1992; Graham et al. 1995; Lai and Lemke, 1991). Single, double, and even triple knockouts are viable without obvious signs of

developmental defect at birth (Lemke and Lu 2003; Lu and Lemke 2001; Lu et al. 1999). These data suggest that the TAM RTKs are largely non-essential for embryogenesis. In adult tissues, Tyro-3, AXL, and Mer exhibit widespread distribution with overlapping but unique expression profiles. Tyro-3 is abundantly expressed in the nervous system, and is also found in ovary, testis, breast, lung, kidney, osteoclasts, and retina as well as in a number of hematopoietic cell lines including monocytes/macrophages and platelets (Angelillo-Scherrer et al. 2001; Lai et al. 1994; Lu and Lemke 2001; Prasad et al. 2006). AXL is expressed ubiquitously (O'Bryan et al. 1991), with notable levels found in the hippocampus and cerebellum (Bellosta et al. 1995) as well as monocytes/macrophages, platelets, endothelial cells, heart, skeletal muscle, liver, kidney, and testis (Angelillo-Scherrer et al. 2001; Graham et al. 1995; Neubauer et al. 1994). Within the hematopoietic lineages, Mer is expressed in monocytes/macrophages, dendritic cells, NK cells, NKT cells, megakaryocytes, and platelets (Angelillo-Scherrer et al. 2001; Behrens et al. 2003; Graham et al. 1994). High levels of Mer expression are also detected in ovary, prostate, testis, lung, retina, and kidney, while lower levels of Mer are found in heart, brain, and skeletal muscle (Graham et al. 1994, 1995; Prasad et al. 2006).

#### 1.1.1.3 *Functions*

Functional studies have shown that the TAM receptors play an important role in the immune response by regulating the phagocytosis of apoptotic cells (Lemke and Burstyn Cohen 2010), the direct suppression of the inflammatory response (Rothlin et al. 2007), and the differentiation of natural killer cells (Caraux et al. 2006). In addition to their ability to regulate the immune response (Rothlin and Lemke 2010), these receptors have also been implicated in blood coagulation (Hafizi and Dahlback 2006), reproduction (Wu et al. 2008), diabetic nephropathy (Arai et al. 2008), and CNS function (Binder and Kilpatrick 2009). In addition, Mer physiological role in clearance of retinal rods- and cones-shedded outer segments was described by functional ablation of Mer that resulted in the development of retinitis pigmentosa (Duncan et al. 2003; Camenisch et al. 1999).

#### 1.1.1.4 *TAM Receptors and Cancer*

In addition to the normal expression profile Tyro-3, AXL, and Mer also display ectopic or overexpression singly or in combination in numerous cancers, including myeloid and lymphoblastic leukemias, melanoma, breast, lung, colon, liver, gastric, kidney, ovarian, uterine, thyroid and brain cancers. Preclinical evidence indicates prominent involvement of Gas6 and TAM receptors in the pathobiology of cancer. Therefore, it is not surprising that different TAM receptors and Gas6 are overexpressed in different human tumor cell lines as well as in primary cancer tissues (Hafizi and Dahlback 2006; Linger et al. 2011). Several studies link expression levels of Gas6 and AXL to prognosis of cancer patients, while only scarce data exist on the prognostic impact of Tyro3, Mer and protein S. Furthermore, AXL mediates resistance towards chemo- and targeted therapy including anti-VEGF or anti-EGFR therapy (Ye et al. 2010). Tyro3 can transform cells in vitro, but its role in cancer is not well-defined like that of AXL. In particular, Tyro3 can promote malignancy by inducing proliferation, which is at least partially mediated via PI3K signaling (Lan et al. 2000). Mer is also involved in tumor biology, but data on this topic is still scarce. Knocking down Mer in astrocytoma cell lines increases apoptosis and similar to AXL, Mer signals through p-Akt and through p-Erk1/2.

As already mentioned Gas6 is a common ligand for all 3 TAM receptors with different affinities (AXL>Tyro3>Mer). The major source of Gas6 is Tumor Associated Macrophages. Tumor cells in turn stimulate macrophages to upregulate Gas6 by expressing IL-10 and M-CSF, thereby inducing a vicious circle (Fig.2), because Gas6 through TAM receptors can foster cancer by promoting different hallmarks of malignancy. The protumoral action of the Gas6-TAM receptor axis has been recognized, and disruption of this axis might also open up novel avenues in cancer treatment (Schmidt et al. 2012). Furthermore, there are reports suggesting that AXL acquires ligand-mediated activation through the intrinsic presence of ligand Gas6 (Avilla et al. 2011). Though all three RTKs have a recognized role in tumorigenesis, it is AXL that has been established as potential cancer target, due to its over expression in several types of cancers (Graham et al. 1994) and its involvement in drug resistance.

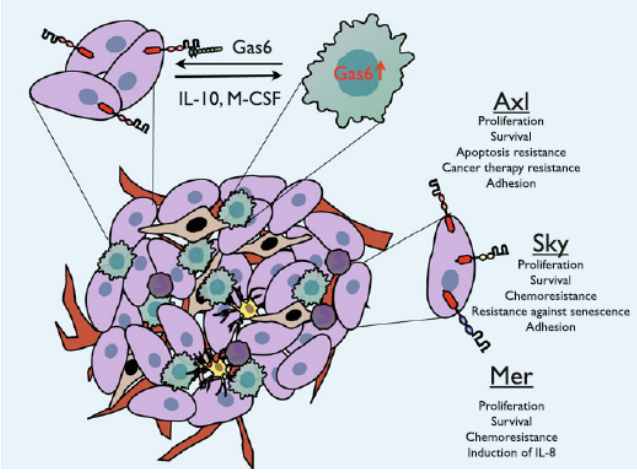


**Figure 2**. A Paracrine mode of TAM receptor activation (adapted from Schmidt et al. 2012)

## 1.2 AXL Receptor Tyrosine Kinase

The *AXL* gene localized at $19q^{13.1\text{-}13.2}$ (O'Bryan et al. 1991; Janssen et al. 1991), and it is known to have two splice variants, whose products are slightly different in size and molecular weight, resulting from alternative splicing of exon 10 and exon 11 (O'Bryan et al. 1991). Isoform one has nine amino acids more than isoform two. These amino acids are located within the ectodomain outside the transmembrane region on the carboxy-terminal side of the FNIII domains. Isoform two lacks exon ten coding for these nine amino acids. The full-length AXL protein contains 885 or 894 amino acids corresponding to the two isoforms, and the predicted molecular weight of AXL is about 98 KDa. However, three different forms resolve in SDS PAGE, i.e., 140, 120 and 104 KDa that presumably correspond to fully-, partially-, and non-glycosylated forms, respectively (O'Bryan et al.1991). AXL obtained its name from the Greek word *"Anexelekto"* meaning "uncontrolled", and its transforming potential in human cancer has been suggested to be a result of overexpression rather than genetic alterations (O'Bryan et al.1991). Gas6 is the known and established ligand for AXL (Varnum et al. 1995).

### 1.2.1 AXL in Cancer Pathogenesis and Progression

AXL was originally identified as a transforming gene involved in the progression of chronic myeloid leukemia (CML) to acute myeloid leukemia (AML) (Liu et al. 1988) and in chronic myeloproliferative disease (Janssen et al. 1991). AXL has been shown to be overexpressed and to have mitogenic

and prosurvival roles in a broad spectrum of human malignancies, which has been summarised in Table 1.

**Table 1**.Malignancies showing AXL overexpression (Modified from Verma et al. 2011).

| S.NO | CANCER TYPE | REFERENCE |
|---|---|---|
| 1 | Acute leukemia (ALL, AML) | Rochlitz et al. 1999; Hong et al. 2008 |
| 2 | Astrocytoma | Hutterer et al. 2008; Vajkoczy et al 2006 |
| 3 | Breast cancer | Berclaz et al. 2001; Gjerdrum et al. 2010 |
| 4 | Colorectal Carcinoma | Craven et al. 1995 |
| 5 | Esophageal Adenocarcinoma | Hector et al. 2010 |
| 6 | Gastrointestinal stromal tumors | Mahadevan et al. 2007 |
| 7 | Gastric cancer | Wu et al. 2002 |
| 8 | Hepatocellular carcinoma | He et al. 2010 |
| 9 | Kaposi sarcoma | Liu et al. 2010 |
| 10 | Lung cancer | Shieh et al. 2005; Linger et al. 2012 |
| 11 | Melanoma | Quong et al. 1994 |
| 12 | Ovarian cancer | Rankin et al. 2010 |
| 13 | Osteosarcoma | Nakano et al. 2003 |
| 14 | Pancreatic ductal adenocarcinoma | Koorstra et al. 2009 |
| 15 | Renal cell carcinoma | Gustafsson et al. 2009 |
| 16 | Prostate cancer | Sainaghi et al. 2005; Paccez et al. 2013 |
| 17 | Thyroid cancer | Ito et al. 1999; Avilla et al. 2011 |
| 18 | Uterine endometrial cancer | Sun et al. 2003 |

Both AXL and its ligand Gas6 are frequently overexpressed and predict poor prognosis (Hutterer et al. 2008). Moreover, AXL affects multiple cellular behaviors required for neovascularization, such as endothelial proliferation, migration, survival, and tube formation *in vitro*, and regulates angiogenesis *in vivo* (Holland et al. 2005). Though AXL is a transmembrane protein, a soluble form of AXL (sAXL), consisting of the proteolytically released extracellular

domain is present in human serum (Budagian et al. 2005; Balogh et al. 2005). Gas6 is also present at low concentrations in circulation, and in both mouse and human serum Gas6 circulates in complex with sAXL (Budagian et al. 2005; Balogh et al. 2005). The TAM receptor involvement in thyroid carcinoma with prime focus on the tumor driving ability of AXL was described (Avilla et al.2011). This particular study showed that human PTC/ATC cells and samples, but not normal thyroid, constitutively express AXL. Moreover, the critical role of AXL in mediating thyroid cancer cell proliferation, survival, invasiveness and experimental tumor growth formation in nude mice was demonstrated, suggesting AXL and its signalling axis, as a potential therapeutic target in thyroid carcinoma. To date, no activating mutations in AXL have been reported in cancer; however, dysregulation of AXL-mediated signaling pathways plays an important role in oncogenic transformation.

### 1.2.2 AXL mediated signalling pathways

AXL is a proto-oncogene whose overexpression can transform cells even in the absence of ligand, and it is believed that AXL transforming potential is due to activation and overexpression of the receptor rather than any genetic alterations (O'Bryan et al. 1991; Janssen et al. 1991). AXL activation in tumor cells has been suggested to follow an autocrine/paracrine pathway (Dirks et al. 1999; Avilla et al. 2011), and AXL transforming capacity also seem to be cell-specific (Burchert et al. 1998). Gas6 activation of AXL results in phosphorylation of three known consensus tyrosine residues kinase region that recruit various signaling molecules: tyrosine 779 (pY*ALM*; phosphatidylinositol 3-kinase (PI3K) binding site), 821 (pY*VNM*; PLCγ-, PI3K-, c-Src-, Lck-, Grb2-binding site), and 866 (pY*VLC*-, PLCγ-binding site) (Braunger et al. 1997; Weinger et al. 2008). Activation of AXL stimulates both proliferative and anti-apoptotic signalling, including PI3K/AKT and MAPK/ERK pathways (Linger et al. 2008). Several lines of evidence suggest that anti-apoptotic effect are the most relevant effects of Gas6-mediated AXL signaling (Goruppi et al. 1996; 1997; Melaragno et al. 2004). It is speculated that AXL-Gas6 axis can generate two different context-dependent PI3K signaling pathways; one where PI3K binds directly to AXL resulting in survival, and one where PI3K binds indirectly to AXL through Grb2 leading to proliferation (Weinger et al. 2008). However Ras/Erk axis mediated growth and proliferation has also been reported (Fridell et al. 1996; Stenhoff et al. 2004; Sainaghi et al. 2005).

## 1.3 Targeting a Tyrosine Kinase Receptor

RTK activation regulates many key pathways leading to malignant phenotype; consistently, dysregulation of RTKs has been shown to correlate with the development and progression of numerous cancers. Therefore RTKs have become an attractive therapeutic target. On a broad view there are two types of RTK targeting approach, using agents with a capability of targeting one or a few targets and using those with a broad one (multiple targets). The first two approaches are carried out using Small Molecule Inhibitors: E.g. Imatinib (Gleevec), which targets the ATP binding site of the kinases, or Antireceptor Antibodies: E.g. Herceptin (Trastuzumab), which targets HER2 (Fig.3). Both these approaches hold their own positives and negatives. For instance, targeting a single target has been successful when the target controls the majority of the critical oncogenic signalling. However, this narrowed targeting is effective only for a short-term, as several clinical studies have shown the resistance acquired to the single targeting agent. On the other hand, agents targeting multiple pathways simultaneously are more effective than agents with narrow selectivity, they also decrease the likelihood of resistance development, but the toxicity and side effects in this approach are generally more than the previous one.

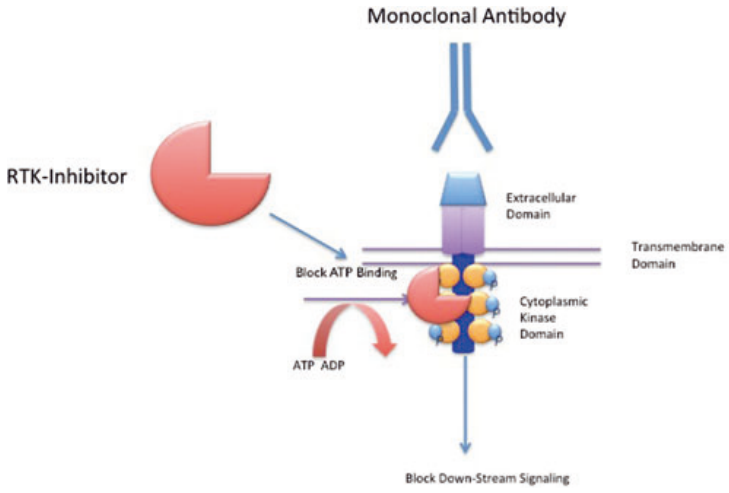


**Figure3**: Strategies to target RTK's (adapted from Bavcar and Argyle 2012)

With the rapid rise of tumor resistance, combinatorial anticancer therapies have gained favour over single molecule inhibition. In this regard, HSP90 inhibitors have rapidly emerged as a class of promising drugs that can target multiple oncogenic pathways simultaneously. HSP90 is a highly

conserved chaperone protein involved in essential cellular functions such as protein folding and cell signalling in both stressed and unstressed cells. In the past decade, a large number of oncogenic client proteins have been identified to associate with HSP90 and contribute to malignant transformation, in which receptor tyrosine kinases are the notable ones. Hence, oncogenic RTKs that are client of HSP90 are targetable through inhibiting HSP90.

Several reports documented the dependence of cancer cells on molecular chaperones, such as Hsp70 and Hsp90, and this notion has added chaperone inhibitors to the arsenal of anticancer drugs (de Billy et al. 2009; Whitesell and Lindquist 2009). Hsp90 inhibitors are in fact already in clinical trials, with promising results (Taldone et al. 2008; Hwang et al. 2009).

### 1.4 Molecular Chaperones

Cells harbour a set of proteins called molecular chaperones which assist folding and prevent the aggregation of misfolded proteins during *de novo* protein synthesis as well as under stress conditions such as high temperature (Young et al. 2004). They are ubiquitous proteins and are life-guards of the living cells by preventing the protein misfolding and aggregation, which is otherwise toxic to the cell. Molecular chaperones perform basic and essential cell functions, such as facilitating newly synthesized polypeptides to fold properly into their biologically native structure, assisting denatured protein in refolding, and preventing protein aggregation. Misfolding of any cellular protein usually results in the exposure of hydrophobic surfaces that in its native state are normally buried in the interior. Hydrophobic surfaces when exposed tend to associate with those from other proteins generating aggregation. Molecular chaperones bind such exposed surfaces through their own exposed hydrophobic binding sites and assist in refolding. Molecular chaperones increase the yield of folding reactions by amplifying the number of productive folding pathways (Frydman 2001; Hartl 1996; Buchner 1996).

Chaperones are divided into four major families, Hsp90, Hsp70, Hsp60, and Hsp20, based on their relative molecular mass (Calderwood et al. 2006; Jego et al. 2010), while additional novel families include Hsp110 and Hsp170 (Easton et al. 2000). To date, more than 200 HSP client proteins have been identified involving nearly all fundamental cellular activities and processes, including cell growth, proliferation, and cell survival (Jego et al. 2010). Interestingly, many cancer-associated proteins have been reported as HSP clients, likely as a mechanism for promoting oncogenic transformation. Moreover, in order to survive under the harsh conditions within the tumor

microenvironment, cancer cells typically become dependent on stress-inducible HSP's. Therefore, targeting HSPs would result in simultaneous inhibition of multiple signaling pathways responsible for modulation of various events involved in cancer progression for a broad range of tumor types, such as neoplastic growth, sustained angiogenesis, chemotherapeutic resistance, evasion of cell death, and ultimately, invasion and metastasis (Barginear et al. 2008).

### 1.4.1 Heat Shock Protein 90

The HSP90 is an ubiquitously expressed cellular protein that constitutes 1–2% of the total protein load (Csermely et al. 1998; Crevel et al. 2001). Five HSP90 isoforms have been identified so far that include cytoplasmic HSP90$\alpha$- and $\beta$-isoforms, endoplasmic reticulum localized glucose regulated protein 94 (GRP94), mitochondrial tumor necrosis factor receptor-associated protein 1 (TRAP1) and membrane-associated HSP90N (Pearl and Prodromou 2000). Eukaryotic Hsp90 chaperones are the key players in the signal transduction networks of the cell, interacting with numerous client substrates in a complex system together with Hsp70 chaperones and a large number of co-chaperones.

### 1.4.2 Hsp90 Structure

The HSP90 protein consists of three highly conserved domains: an N-terminal nucleotide binding domain (NBD), a middle domain (MD) and a C-terminal dimerization domain (DD) necessary to form Homodimeric complexes (Fig.4).

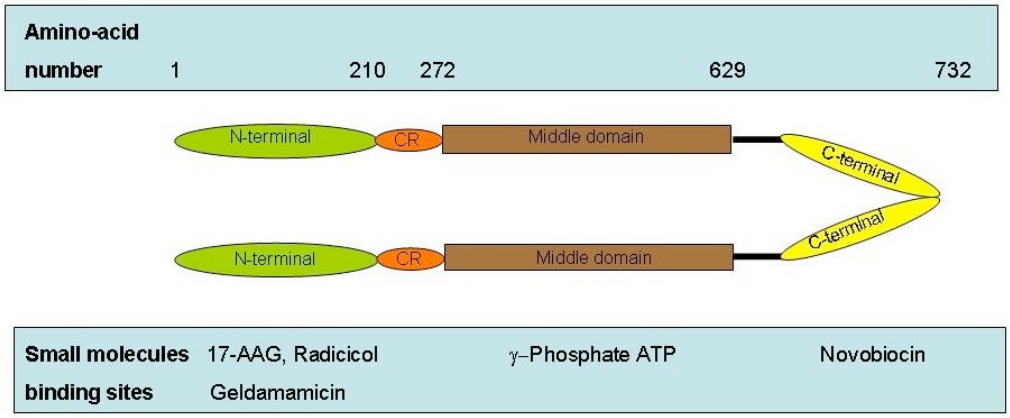

**Figure 4**: Structure of the Hsp90 dimer. The numbering 1–732 indicates the approximate positions in the amino acid sequence of the human protein that define its functional domains. 'CR' refers to a charged region which serves as a flexible linker between the N-terminal and middle domains. The locations where ATP and inhibitors (small molecules) bind Hsp90 and modulate its function are indicated.

Three-dimensional crystallization structures revealed a two layer α/β sandwich structure in which the helixes delimit a pocket extending from the surface to the buried face of highly twisted antiparallel β–sheet. In the human structure, this pocket was found to be the binding site for ATP and for the macrocyclic antitumour agent geldanamycin, whose binding to Hsp90 had been shown to disrupt productive complexes of Hsp90 with client protein such as kinases and steroid hormone receptors (Whitesell et al. 1994; Chavany et al. 1996). The middle domain consists of a large N-terminal αβα (αhelix-βsheet-αhelix) domain connecting to a small αβα domain at the C-terminal via series of short α–helixes in a tight coil. The middle segment is the major site for client protein interactions, with a conserved hydrophobic patch centred on Trp 300 and an unusual amphipatic protrusion formed by residues 327-340. The middle segment also contributed a key catalytic lysine residue that would interact with the γ–phosphate of an ATP molecule bound in the N-terminal domain (Meyer et al. 2003). The C-terminal domain is essential for Hsp90 dimerization. The dimer interface is formed by a pair of helixes at the C-terminal end of the domain packing together to create a four-helix bundle. The Cterminal domain presents the MEEVD motif implicated in binding to cochaperones with tetratricopeptide repeat (TRP) domains (Young et al. 1998).

Co-chaperones, such as HOP, CDC37, p23, Aha1 and PPIase, play an important role in client protein maturation and modulation of ATPase activity. Co-chaperones also recruit specific client proteins to HSP90 and/or stabilize HSP90 in an ATP-bound state to prolong the half-life of the mature multi-chaperone complex (Wandinger et al. 2008). ATP hydrolysis alters HSP90 structure and promotes its chaperone function. Therefore, in cells, HSP90 generally exists in 2 conformations – an active "open" conformation that is in an ATP bound state during client protein binding, and an inactive "closed" conformation (Buchner 1999).

### 1.4.3 Hsp90 chaperone cycle

Hsp90 function in client activation and maturation is highly dependent on binding and hydrolysis of ATP (Obermann et al. 1998; Panaretou et al. 1998) which drives a dynamic conformational cycle that involves interactions

with the Hsp70 system and an increasing number of co-chaperones. The HSP90 chaperone cycle consists of transitions between open and closed protein conformations (Kamal et al. 2004). Throughout each cycle, the transition between open and closed states allows for the rearrangement of different co-chaperones that associate with HSP90. To begin the cycle, the two N-terminal domains of the dimer are in a separated, open state, which is maintained through interaction with Hop at both the N-terminal and C-terminal domains (Richter et al. 2008; Prodromou et al. 1999; Richter et al. 2003). A client protein is recruited to HSP90/HOP via the HSP70/HSP40 co-chaperone complex, which associates with HSP90 through the interaction of HSP70 with Hop (Pearl et al. 2008). While in this intermediate complex, the client protein is transferred from HSP70 to the middle domain of HSP90. At this point, ATP is bound to each of the N-terminal binding pockets. Next, Hop disassociates from the HSP90 complex, due to reduced affinity following binding of ATP and the co-chaperone p23 (Li et al. 2011; 2012). In this mature complex, the N-terminal and middle domain are brought together to form the closed state of the chaperone complex. At this stage, both HSP90 and p23 facilitate folding of the client protein (Li et al. 2011; 2012). To complete the cycle, additional co-chaperones, such as aha1 (activator of HSP90 ATPase) and the peptidylprolyl cis-trans isomerases Cpr6/Cyp40, bind to the chaperone complex and induce ATP hydrolysis (Panaretou et al. 2002; McLaughlin et al. 2002). Conversion of ATP to ADP + Pi allows the HSP90 dimer to reopen, and the newly or refolded client protein disassociates from the chaperone complex. Upon returning to its open state, HSP90 reassociates with Hop in place of p23, and thus completes the cycle (Hessling et al. 2009).

In particular, HSP90 role in the folding and maturation of various client proteins, as well as the renaturation of misfolded proteins (Shen et al. 2005; Chiosis et al. 2001), makes them potential targets for many diseases ranging from the disruption of multiple signaling pathways associated with cancer to the clearance of protein aggregates in neurodegenerative diseases. In fact, inhibitors of Hsp90 are the only cancer chemotherapeutic agents known to disrupt all six hallmarks of cancer simultaneously (Guo et al. 2006). Previous studies show that level of Hsp90 is higher in various malignant cell lines and cancer cells compared to normal cells (Whitesell and Lindquist 2005).

### 1.4.4 Hsp90 - A Potential Cancer Therapeutic Target

HSP90 function maintains the cancer phenotype by helping cancer cells to overcome environmental stress, proteotoxic stress, nutrient insufficiency,

redox state, and the necessity to escape from immune destruction (Neckers and Workman 2012). Many of its client proteins are signaling oncoproteins that represent nodal points in multiple oncogenic signaling pathways (Modi et al. 2011; Neckers and Workman 2012). Examples of client proteins of HSP90 that play an important role in cancer include the human epidermal growth factor receptor 2 (HER2), mutant epidermal growth factor receptor (EGFR), the BCR-ABL fusion protein, BRAF, the serine-threonine protein kinase AKT/PKB, c-MET, as well as the steroid hormone receptors (estrogen receptor and androgen receptor). The selected examples of HSP90 client protein pathways involved in sustaining cancer phenotype is shown in Figure 5. Other than mediating the oncogenic signalling cascade through chaperoning the signal transducing oncoproteins, HSP90 also involved in other hallmark processes of cancer, including induction of angiogenesis, resistance to cell death (anti-apoptosis), and promotion of metastasis. For instance, HSP90 influences angiogenesis by chaperoning hypoxia inducible factor-1α (HIF-1 alpha) and vascular endothelial growth factor receptor (VEGFR), in addition to governing nitric oxide synthase upregulation. HSP90 chaperones client proteins that are anti-apoptotic, including AKT and survivin. Thus, inhibition of HSP90 could lead to increased apoptosis and enhanced anticancer activity. Also, HSP90 chaperoning promote metastasis through matrix metalloproteinase-2 (MMP-2) activation (Hanahan and Weinberg 2011; Basso et al. 2002). Other client proteins of HSP90 that play a role in cell signaling processes include IL6R (JAK/STAT pathway), FAK (integrin pathway), CDK 4, 6, 9 (cell cycling), IkB kinases (NFkB pathway) and APAF-1 (apoptosis) (Khong and Spencer 2011).

The role of HSP90 in malignancy is mediated by its ability to control the stabilization of its oncogenic client proteins, as well as regulate their activated states. Cancer cells that depend on these HSP90 dependent oncoproteins are sensitive to HSP90 inhibition (Workman and Powers 2007). Thus blockade of many key cancer causing pathways upon the disruption of the HSP90 chaperone complex association with the client, results in client proteins polyubiquitination and proteasome-dependent degradation (Neckers et al. 1999; Xu et al. 2005). Recent studies indicate that combination of low dose Hsp90 inhibitors with conventional chemotherapeutic agents or radiation provides an effective strategy in cancer therapy. One concern is that HSP90 inhibitors may act as inducers of all heat shock proteins, including Hsp40 and Hsp70, *via* activation of heat shock factor 1 (HSF-1) (Bagatell et al. 2000; Bagatell and whitesell 2004). Therapies that combine Hsp90 and Hsp70

inhibition, for instance, might be necessary to improve efficacy of either treatment alone (Guo et al. 2005).

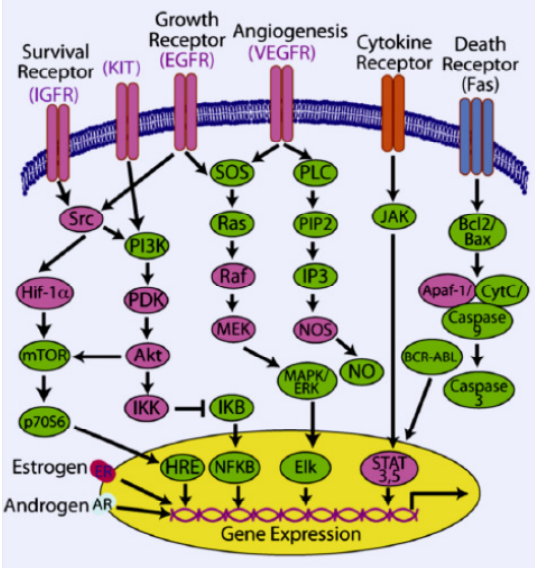


**Figure 5**: Selected examples of HSP90 client protein pathways involved in survival and anti-apoptosis of tumor cells. JAK – Janus family of tyrosine kinases; STAT – Signal transducers and activators of transcription; MAPK/ERK – Mitogen activated protein kinase, Extracellular signal-regulated kinase; PDK – Phosphoinositide-dependent kinase. (Hong et al. 2012 Cancer Treatment Review)

## 1.5 HSP90 Inhibitors

As already mentioned, HSP90 chaperone consists of three domains: (i) the amino terminal region (N-domain) that contains the ATP, drug-binding site, and co-chaperone interacting motifs, (ii) a middle (M) domain that participates in forming active ATPase and also serves as a docking site for client proteins and cochaperones, and (iii) the carboxy terminal region (C-domain) that contains a dimerization motif, a second drug-binding site, and interaction sites for cochaperones (Ali et al. 2006; Prodromou & Pearl 2003). Thus far, all the HSP90 inhibitors in clinical trials work by inhibiting the ATPase activity of HSP90 by binding the drug-binding site on the N domain (Janin 2010; Taldone et al. 2009). In general, they can be classified into first-generation inhibitors, based on their similarity to Geldanamycin, or second-generation small molecule synthetic inhibitors like resorcinol or purine derivatives.

### 1.5.1 Geldanamycin (GA) Derivatives - The First-Generation HSP90 Inhibitors

Geldanamycin (GA) is an ansamycin antibiotic first isolated from streptomyces hygroscopicus (DeBoer et al. 1970). The effects observed from the naturally occurring compound has opened the gateway for HSP90 inhibitor development, as this compound showed destabilizing effects on some HSP90 clients in the preclinical studies (Workman et al. 2007). It has been demonstrated through cocrystallographic study that GA competes with ATP binding to the nucleotide binding pocket on the N-terminal domain of HSP90, thereby inhibiting its ATPase activity (Stebbins et al. 1997; Sittler et al.2001). As Geldanamycin exhibited severe hepatotoxicity, its use in the clinic was discontinued. However, derivatives of geldanamycin proved better pharmacological properties and successfully entered into clinical trials as first HSP90 inhibitors. The primary analogs of GA include Tanespimycin (17-allylamino-17-demethoxygeldanamycin, 17-AAG), Alvespimycin (17-dimethylaminoethylamino-17-demethoxygeldanamycin, 17-DMAG), retaspimycin or IPI-504 (17-Allylamino-17-Demethoxygeldanamycin Hydroquinone Hydrochloride).

#### 1.5.1.1 *Tanespimycin*

17-allylamino-17-demethoxygeldanamycin (17-AAG), a geldanamycin derivative, was the first HSP90 inhibitor to be evaluated in humans. 17-AAG was derived by substituting the nonessential methoxy group on the C-17 of the quinine ring in GA with an amino group. 17-AAG retained the antitumor properties of GA with a more favourable and acceptable safety profile (Solit et al. 2002; de Candia et al. 2003). 17-AAG entered clinical trials in cancer patients in the US and UK (Sausville et al. 2003; Goetz et al. 2005). Although initial trials were disappointing, development of an improved formulation of 17-AAG by Kosan Pharmaceuticals named Tanespimycin (KOS-953), displayed a promising clinical relevance. Under this new formulation, impressive clinical activity were noted in trastuzumab-refractory HER2-positive breast cancer and in multiple myeloma, especially in bortezomib refractory patients (Modi et al. 2011). These data prompted a phase III trial of tanespimycin plus bortezomib for this indication (ClinicalTrials.gov identifier: NCT00514371). Despite its promising activity in clinical studies, the presence of the benzoquinone sub-group in the structure of tanespimycin may contribute to the drug's greater hepatic toxicity, and constitute a mechanism of drug resistance in patients with mutated/altered NQO1 (Neckers and Workman

2012). Indeed, tanespimycin's clinical limitations are modest bioavailability, instability and toxicity (Workman et al. 2007).

#### 1.5.1.2 *Alvespimycin*

17-Desmethoxy-17-N,N-Dimethylaminoethylaminogeldanamycin (17-DMAG) was formed as a result of substituting the C-17 methoxy group of GA with N-N-dimethylethylamine. This formulation has increased water solubility and equal or better potency compared to 17-AAG (Hollingshead et al. 2005). This is the second HSP90 inhibitor to enter clinical trials and is less sensitive to and less dependent on NQO1 with better pharmacokinetic properties (Neckers and Workman 2012) and fewer side-effects (Workman et al. 2007; Li et al. 2009). Alvespimycin retained tanespimycin's activity, or even greater. In a study of alvespimycin administered to 25 patients with advanced malignancies, complete response (CR) occurred in one patient with castration resistant prostate cancer (CRPC), partial response (PR) in one patient with metastatic melanoma, and stable disease (SD) >6 months occurred in three patients with chondrosarcoma, CRPC and clear cell renal cancer respectively (Pacey et al. 2011). However, alvespimycin, like tanespimycin, may be most beneficial in combination with client protein inhibiting drugs, such as trastuzumab. A recent phase I study of such combination showed a partial response in a HER2+ breast cancer patient and stable disease >6 months in 6/28 Patients (Jhaveri et al. 2012).

#### 1.5.1.3 *Retaspimycin*

IPI-504 is a water soluble, hydroquinone hydrochloride salt derivative of 17-AAG, and the hydroquinone form (IPI-504) is in redox equilibrium with the quinone form (17-AAG). IPI-504, when subjected to phase II trial in refractory NSCLC showed a promising response rate in patients having ALK rearrangement but not in those without ALK rearrangement (Sequist et al. 2010). The high degree of sensitivity of EML4-ALK to HSP90 inhibition by IPI-504 suggests that patients having cancer subtypes with EML4-ALK as a key oncogenic driver may be more sensitive to HSP90 inhibition (Normant et al. 2011). Promising activity was also seen in GISTs, which formed the basis of the phase III RING (Retaspimycin in GIST) trial. Regardless of the antitumor effect seen, due to the high mortality rate in the IPI-504 treatment arm, this trial was recently suspended. IPI-493, a similar derivative in oral form, has entered in phase I study.

### 1.5.2 Synthetic Small Molecules – The Second and Third Generation HSP90 Inhibitors

Synthetic molecules have been designed to evade the toxic side effects attributed to the benzoquinone group on geldanamycin-based molecules. These compounds bind the N-terminal ATPase site of HSP90 with higher affinity than the natural nucleotides and prevent HSP90 from cycling between its ADP- and ATP-bound conformations. The first group of these agents is the purine scaffold series, based on the prototype PU3 developed by the Chiosis laboratory (Chiosis et al. 2001). Purines such as CNF2024/BIIB021, MPC-3100, and PU-H71 and purine-like analog Debio-0932 (CUDC-305) demonstrate insensitivity to multidrug resistance, high aqueous solubility, and oral bioavailability (Chiosis et al. 2002; Rodina et al. 2007). CNF2024, an orally available 9-benzyl purine derivative developed by Conformal Therapeutics, following its clinical activity and favourable tolerability in the phase I trials in chronic lymphocytic leukemia (CLL), lymphomas, advanced solid tumors, and most recently in breast cancer (Jhaveri et al. 2012) has entered phase II studies.

Several other small molecule HSP90 inhibitors have recently been reported in the clinical setting including AUY922, AT13387, STA9090, and MPC3100. In a phase I study involving treatment with AUY922, extended disease stabilization in a subset of advanced solid tumor patients was noted (Samuel et al. 2010). AT13387 in vitro has been the longest-acting HSP90 inhibitor to date, suppressing client proteins longer than 7 days. Beside these, a new class of compounds that target HSP90's C-terminal domain have been developed, and investigators have been able to increase the affinity of these molecules for HSP90. A family of coumarin antibiotics, the most notable of them being DB01051 (novobiocin), binds to the C-terminal domain of HSP90 and disrupts the HSP90 chaperone complex, resulting in HSP90 client protein degradation similar in action to the compounds that bind to the N-terminus, but with less degradative ability (Marcu et al. 2000). Advantages of HSP90 inhibitors that target the C-terminal domain (C-terminal inhibitors) include less robust activation of heat shock factor 1 (HSF1), which induces HSP90 transcription. However, concentrations of novobiocin high enough to inhibit HSP90 have not been reached in vivo, thus several other derivatives of novobiocin such as triazole containing analogues are in development (Peterson and Blagg 2010).

# 2. AIM OF THE STUDY

AXL plays a significant role in pathogenesis and progression of human cancer and in anti-cancer drug resistance. Our previous observations that AXL is deregulated in Thyroid cancer and its inhibition results in decreased proliferation, invasiveness and experimental tumor growth formation in nude mice supports several other reports described for different types of cancers.

HSP90 is a critical player in maintaining the cancer phenotype by its ability to control the stability of its oncogenic client proteins. HSP90 inhibition causes depletion of multiple oncogenic client proteins, leading to the blockade of many key cancer causing pathways. The greatest therapeutic benefits with HSP90 inhibitors could derive from targeting tumors that have its client proteins as oncogenic drivers.

On aiming to devise a strategy to target the receptor tyrosine kinase AXL, we opted to use the HSP90 inhibition approach based on our observation that HSP90 inhibitors effectively downregulate AXL protein levels. This thesis will contribute significantly in validating AXL as a novel client of HSP90, an ongoing target for the treatment of cancer.

In particular, the major focus of this thesis will address the following topics:

1. Validation of AXL as a Novel client of HSP90.

2. Molecular mechanism of 17-AAG induced AXL degradation.

3. The effect of 17-AAG on AXL driven signalling and biological activity.

# 3. MATERIALS AND METHODS

## 3.1 Reagents and Antibodies

17-AAG, Radicicol and MG132 were purchased from Calbiochem (Merck KGaA, Darmstad, Germany). Lactacystin, Ammonium chloride, and Chloroquine were purchased from Sigma (St. Luis, MO, USA). Endo H and Peptide-N-Glycosidase (PNGase) were from New England Biolabs (Ipswich, MA, USA). Anti- HSP90 (#SPA-835) and anti-HSP70 (#SPA-810) were purchased from Stressgen biotechnologies (Victoria, BC, Canada). Anti-AXL, anti-Tyro3, anti-myc, anti-HA antibodies used in western blot and immunoprecipitation experiments were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A). The anti-AXL antibody directed to AXL extracellular domain and anti-phospho AXL, specific to tyrosine 779, were from R&D Systems (Abingdon, UK). Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology (Lake Placid, NY, USA) and anti-DYKDDD tag (FLAG epitope) antibody from Cell signalling Technologies (Beverly, MA, USA). Secondary antibodies coupled to horseradish peroxidase were from BioRad (Munchen, Germany).

## 3.2 Cell Lines and Transfection Procedures

HeLa cells were from American Type Culture Collection (ATCC, Manassas, VA). Human thyroid papillary cancer cell line TPC1, and anaplastic thyroid cancer cell lines 8505C and CAL62 have been previously described (Avilla et al. 2011) and were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (Life technologies, GIBCO, Carlsbad, CA, USA). Transient transfections were carried out by premixing each plasmid (1-1.5µg**)** with Fugene6 (Roche, Basel, Switzerland), and the mixture was added to cells at 70% confluency. Cells were continually cultured in the same medium for 48 hours until lysis or further treatments.

## 3.3 Western Blotting and Immunoprecipitation

Protein lysates were prepared according to standard procedures. Cells were lysed at 4ºC in a buffer containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.5), 1% (vol/vol) Triton X-100, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulphonyl fluoride (PMSF), and 1 µg/ml

aprotinin. Lysates were clarified by centrifugation at 10,000 x g for 20 min. Lysates containing comparable amounts of proteins, estimated by a modified Bradford assay (Bio-Rad, Munchen, Germany), were subjected to Western blot. For immunoprecipitation, 1mg of lysate was incubated with appropriate antibody for 3 hrs or overnight and then with the slurry of protein A/G-sepharose for 1 hr at 4ºC. Antigen-Antibody-Bead complexes were centrifuged, washed extensively using wash buffer (20 mM Tris-Hcl pH7.4, 150 mM Nacl, Triton 0.1%), resuspended in Laemmli buffer **(**60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue)**,** boiled, resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with primary antibodies followed by secondary antibodies coupled to horseradish peroxidase. The proteins were detected with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfort, UK).

### 3.4 Plasmids and Constructs

We used pCDNA 4TOA His/myc (Life technologies, Invitrogen, Carlsbad, CA, USA) and pFLAG5a (Sigma) vectors to subclone the full length AXL by PCR amplification. The truncated mutant AXL-EC has been previously described (Avilla et al. 2011), comprises the extracellular and transmembrane domain of the receptor, 1 to 508 amino acids (NM_001699) and it has been obtained from its full length construct by PCR with specific primersF:GAATTCGCTGGGAGCCCAACAACTT, R: AAGCTTCCCA GGCTGTTCAAGGTAGC) and subcloned using ECORI and HINDIII into pFLAG5a vector. PCDNA 3.1 CHIP-myc and CHIP-TPR-myc (K30A) vectors were a kind gift of L. Neckers. PCDNA-HA-Ubiquitin (Ub-HA) vector was a kind gift of S. Giordano. HSP90-HA (García-Cardeña et al. 1998), expressing the Hsp90β wt cDNA in pcDNA3, was obtained from Addgene non-profit plasmid repository, as "Addgene plasmid 22487".

### 3.5 Biotinylation of Surface Proteins

Cells grown around 70% confluence were washed twice with ice-cold PBS with 10 mM $ca^{2+}$ and 1 mM $Mg^{2+}$ and surface proteins were labelled for 30 minutes using 1 mg/ml EZ-link NHS-SS-biotin (Pierce Chemical Co., Rockford, IL). All manipulations were carried out on ice to avoid the internalization at these steps. The unreacted biotin was removed by washing with 50mM $NH_4Cl$ in PBS, and cells were lysed in the RIPA buffer (50 mM Tris-Hcl pH 7.4, 150 mM Nacl, 1% Triton, 0.5% Sodium deoxycholate, 0.1%

SDS and 1 mM EDTA). Comparable amount of proteins were incubated overnight with Streptavidin beads (Pierce Chemical), which were then thoroughly washed, denatured and analyzed by Western blot with anti-AXL antibodies. The assessment of surface protein turnover by surface Biotinylation and chase was performed as previously described (Gottardi et al. 1995), the biotin labelling was performed as above and then cells were allowed to grow under normal condition or with treatment, which are harvested at the appropriate time points.

### 3.6 [$^{35}$S]Methionine labelling and pulse chase analysis of AXL

CAL62 cells plated in 60mm dish at 70% confluence were starved for 1 hr in 1.5 ml of methionine/cysteine-free medium, metabolically labelled with 200 μci of $^{35}$S methionine for 20 mins in methionine/cysteine-free medium (pulse), washed free of unbound radioactive amino acids, and incubated in prewarmed complete medium (chase) in the presence of vehicle or 17-AAG (500 nM). At the indicated time points, the cells were disrupted in ice-cold lysis buffer and comparable amount of whole cell extract were immunoprecipitated with anti-AXL antibody. Proteins were subjected to SDS-PAGE. Labelled proteins were visualized by autoradiography.

### 3.7 Immunofluorescence analysis

To assess the plasma membrane localized AXL through immunofluorescence, cells plated on fibronectin-coated glass coverslips were subjected to 17-AAG treatment for 8 hrs, fixed with 4% paraformaldihyde, blocked using PBS-BSA (1%), incubated with 1:50 dilution of 1μg/μl anti-AXL antibody (R&D Systems) for 2 hrs at room temperature, followed by a 1:1000 diluted rhodamine conjugated anti-goat secondary antibody (Jacksons immunoresearch Europe Ltd, Oaks Drive Newmarket, Suffolk, UK) for 30 min. Analysis was performed using a Zeiss LSM 510 Meta confocal microscope.

### 3.8 Luciferase Activity Assay

Approximately $1\times10^6$ HeLa cells were transiently co-transfected with AXL and the AP1-Luc vector (Stratagene, Garden Grove, CA, USA) containing six AP1-binding sites upstream from the Firefly luciferase cDNA. Twenty-four hours after transfection, cells were serum-starved and the

indicated concentration of 17-AAG or vehicle was added. Cells were harvested 48 hrs after transfection. 10 ng pRL-null (a plasmid expressing the enzyme Renilla luciferase from Renilla reniformis) served as an internal control. Firefly and Renilla luciferase activities were assayed using the Dual-Luciferase reporter system (Promega Corporation, Madison, WI, USA) and expressed as percentage of residual activity compared with cells treated with vehicle. Light emission was measured by using a Berthold Technologies luminometer (Centro LB 960) (Bad Wildbad, Germany) and expressed as ratio of Firefly and Renilla luciferase activities. The ANOVA multiple comparison test was used to assess statistical significance of luciferase assay, and the InStat3 Graphpad Software (La Jolla, CA, USA) was used.

### 3.9 Nickel affinity His-tagged protein purification under denaturing conditions

HeLa cells 48hrs post-transfected with His tagged AXL (sub-cloned in pCDNA 4 TO A His/myc) were harvested and lysed with Buffer A (6 M Guanidium Hcl, 100 mM Sodium phosphate buffer pH.8, 10 mM Tris Hcl pH. 8 and 30 mM Imidazole and 10 mM beta-Mercaptoethanol). The lysate was rocked with Ni-NTA Agarose beads (Qiagen) for 4 hrs to overnight. The beads were collected and washed with Buffer B (8 M Urea, 100 mM Phosphate buffer pH.6.3, 10 mM Tris Hcl pH.6.3, 10 mM beta-Mercaptoethanol and 0.2 % Triton X 100), and eluted in Buffer E (200 mM Imidazole, 150 mM Tris Hcl pH 6.7, 30% Glycerol, 5% SDS and 720 mM Beta-Mercaptoethanol). Proteins were resuspended in sample buffer (2X) and subjected to SDS PAGE followed by western blotting.

# 4. RESULTS

## 4.1 PART I – Validation of AXL as a novel client of HSP90

### 4.1.1 Sensitivity of AXL to HSP90 Inhibitors

A large number of HSP90 client proteins are *bona fide* oncoproteins. These include many kinases such as ERBB2, EGFR, CDK4, CRAF, BRAF, AKT, MET, RET and BCR/ABL, as well as transcription factors such as oestrogen and androgen receptors, HIF-1α and p53. HSP90 inhibition leading to protein destabilization and/or degradation could be considered as a preliminary signature of being the client of HSP90. To test whether AXL protein is downregulated upon HSP90 inhibition, we used a panel of thyroid carcinoma cell lines (CAL62, 8505C and TPC1) and HeLa cells, all endogenously expressing AXL. Cells were exposed to 17-AAG treatment in a dose- and time-dependent manner, and AXL protein levels were detected by western blotting. In all the cell lines tested, 17-AAG induced decrease of AXL protein levels (Fig.6A). AXL protein migrates, on Western blot, as a doublet of 140 KDa and 120 KDa. Interestingly, we observed that 17-AAG induced a strong reduction of the slow-migrating 140 KDa isoform of the receptor, while it caused an accumulation of the 120 KDa band. The same effect was observed on AXL using another HSP90 inhibitor, Radicicol, which is structurally unrelated to 17-AAG (Fig.6B). This effect was specific to AXL as 17-AAG induced degradation of other known HSP90 clients like c-KIT, RET and PDGFR affected equally both the mature and the immature receptor isoforms (Fig.6C).

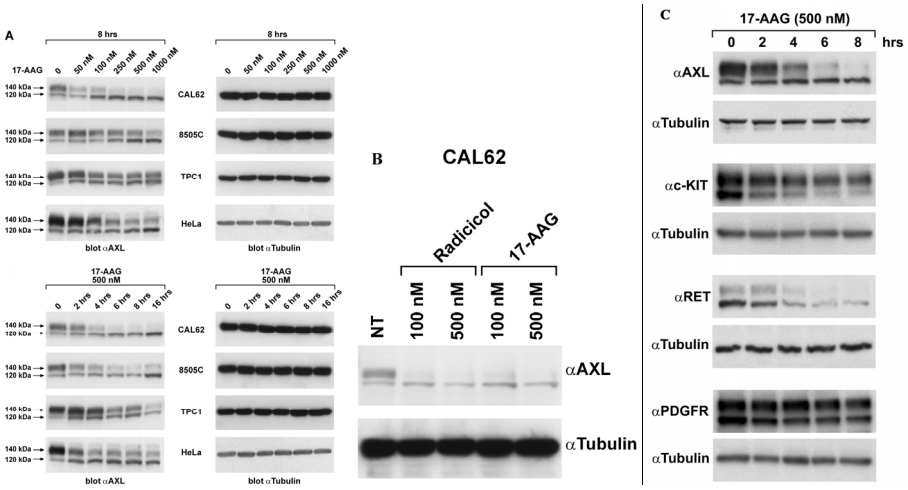

**Figure 6**: (A) Protein lysates from the indicated thyroid carcinoma cell lines (CAL62, 8505C, TPC1) and Hela cells subjected to vehicle (0) or 17-AAG treatment in the indicated doses for 8 hrs (upper panel) and in the fixed dose (500 nM) for indicated time course (lower panel) were immunoblotted using anti-AXL antibodies that recognize both the 140 KDa and the 120 KDa AXL isoforms. Anti-Tubulin antibodies were used as a loading control. (B) CAL62 cells expressing endogenous AXL were treated with the indicated concentrations of Radicicol and 17-AAG for 8 hrs. Equivalent amount of protein lysates were subjected to western blotting with anti-AXL and anti-Tubulin antibodies. (C) HeLa cells transfected with the expression constructs of AXL, c-KIT, RET and PDGFR were treated with 17-AAG (500nM) and harvested at the indicated time points were subjected to SDS PAGE and immunoblotted against the respective protein and Tubulin.

### 4.1.2 17-AAG targets the completely matured AXL receptor

AXL is expected to be glycosylated as its resolving pattern in SDS PAGE shows two bands (140 KDa and 120 KDa), in which the 17-AAG sensitive species of AXL was observed to be the 140 KDa isoform. First, to analyse whether these two bands are glycoslylated, we treated CAL62 cells with the glycosylation inhibitor Tunicamycin. As shown in figure 7A the tunicamycin treatment completely abrogated AXL glycosylation and hence the non-glycosylated isoform of AXL (100 KDa) is the only detected by SDS PAGE/western blotting. This clearly infers that the two AXL isoforms (120 and 140 KDa) are differentially glycosylated forms of the receptor. Second, we proceeded to characterize the band that was sensitive to 17-AAG; to this aim we treated CAL62 cell lysates with two different endoglycosidases, Endo-H and PNGase. These glycosydases are generally used to dissect out the glycosylation status of a glyco-protein. In fact, PNGase removes the whole of N-glycans, whereas EndoH removes only the glycans of high mannose type. The experiment shown in figure 7B clearly depicted the glycosylation pattern of AXL, and confirmed that the band that was sensitive to EndoH was the high mannose type ie., 120KDa isoform; the band that was insensitive to EndoH, but sensitive to PNGase was the completely glycosylated or fully matured isoform (140 KDa). Thus, this experiment clarified that the AXL isoform that was affected by 17-AAG corresponded to the fully glycosylated and completely matured isoform, which is normally localized at the plasma membrane.

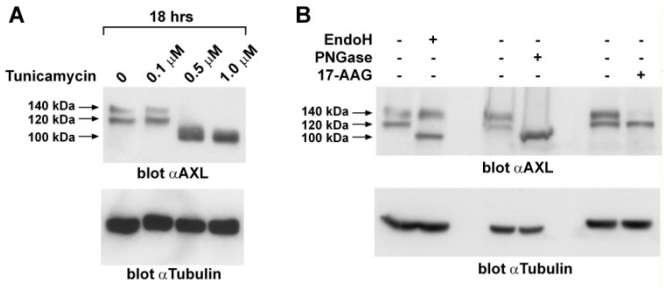

**Figure7**: (A) Lysates of CAL62 cells treated 18 hrs with the indicated doses Tunicamycin were immunoblotted with AXL antibodies and normalised with Anti-Tubulin. (B) 50 µg protein lysates of CAL62 cells pre-boiled for 5 minutes at 99˚C and subjected to overnight EndoH (0.01 U) and PNGase (1 U) digestion at 37 °C. The lysates were run on SDS-PAGE and immunoblotted for anti-AXL and -Tubulin.

To confirm that 17-AAG induced a decrease of the AXL isoform exposed on the cell surface, we performed *in vivo* biotin-labelling of the cell surface proteins in CAL62 cells treated or not with 17-AAG. Biotinylated proteins were recovered with streptavidin-agarose resin and subsequently immunoblotted with anti-AXL antibodies. As shown in figure 8A, the 140 KDa AXL isoform was the only biotinylated product, indicating it normally localizes mainly to the cell surface. No AXL biotinylated product was detected in 17-AAG-treated cells, suggesting that this drug targeted the mature receptor thereby reducing its localization on the plasma membrane. Consistently, immunofluorescence staining on non-permeabilized CAL62 cells treated or not with 17-AAG using an anti-AXL antibody directed to AXL extracellular domain confirmed that 17-AAG treatment induced a strong reduction of surface localized AXL (Fig.8B).

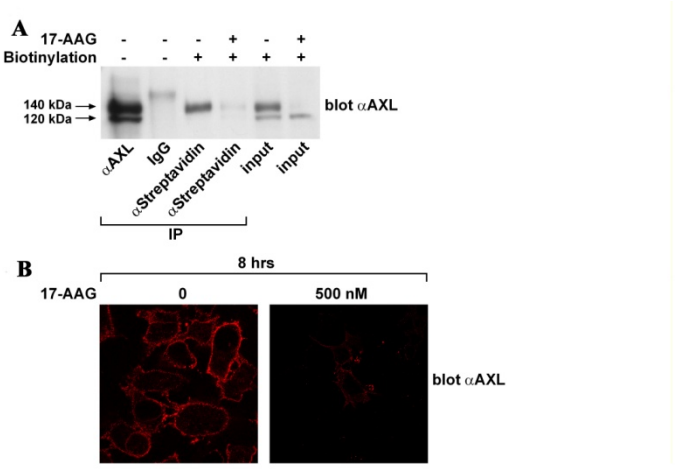


**Figure 8:** (A) CAL62 cells were surface labelled with biotin and treated or not with 17-AAG (500 nM) for 8 hours. Cells were then lysed and 1 mg of lysate was immunoprecipitated using streptavidin-sepharose and immunoblotted for AXL. (B) CAL62 cells, grown on coverslip, were subjected to vehicle (0) or 17-AAG (500 nM) treatment for 8 hrs. Cells were then fixed and stained for immunofluorescence using anti-AXL antibody targeting extracellular domain and a fluorescently labeled secondary antibody to goat immunoglobulins. The slides were analysed under confocal microscope. Representative microscopy images of both treated and untreated cells are presented.

### 4.1.3 Functional interaction of AXL with the chaperone machinery components

We performed co-immunoprecipitation experiments to examine the association between AXL and HSP90 and the crucial components of the chaperone complex in the presence and absence of 17-AAG. HSP90 was co-immunoprecipitated with AXL under basal conditions (Fig.9A). Upon 17-AAG treatment, AXL-HSP90 interaction initially increased but then became undetectable. On the contrary, HSP70 co-immunoprecipitation with AXL was not detectable under basal condition (Fig.9A). However, the exposure of HeLa cells to 17-AAG, besides increasing the cellular concentration of HSP70, also promoted its interaction with AXL; these kinetics of association/dissociation of the chaperones suggest a shift in the chaperoning balance from folding (HSP90) to degradation mode (HSP70) due to the 17-AAG-dependent block of HSP90 activity (Kundrat and Regan 2010).

To validate these interactions and to evaluate HSP90 interaction with the different AXL isoforms, we performed coimmunoprecipitation experiments by co-expressing AXL-Flag and HSP90-HA in HeLa cells. As shown in figure 9B, HSP90 interacted with the high mannose AXL isoform (120 KDa) and the AXL core polypeptide (100 KDa), but not significantly with the fully mature receptor (140 KDa) under basal condition (Fig.9B), despite this particular isoform seemed the most affected upon 17-AAG exposure. This indicated that the exclusive sensitivity of AXL 140 KDa isoform towards HSP90 inhibition could not be explained by the selective interaction with the chaperone. The HSP70-containing chaperone complex destabilizes misfolded proteins by recruiting many different ubiquitin ligases, among which the ubiquitin E3-ligase CHIP was well described (Meacham et al. 2001; Murata et al. 2001). CHIP E3-ligase interacts with HSP70/90 via its amino-terminal tetratricopeptide repeat (TPR) domain (Xu et al. 2002). To test the involvement of CHIP in 17-AAG induced AXL ubiquitination, we co-expressed CHIP-myc and AXL-Flag in HeLa and assessed the co-immunoprecipitation of AXL-Flag in the CHIP-myc immunoprecipitate in the absence and presence of 17-AAG. As shown in Figure 9C, although AXL poorly interacted with CHIP under normal conditions, it robustly associated to CHIP upon 4 hrs of 17-AAG treatment, strongly indicating its functional role in mediating 17-AAG-dependent ubiquitination of the receptor. Surprisingly, the CHIP immunocomplex also displayed a predominant association with the 120 KDa partially glycosylated isoform and with the core polypeptide (100 KDa), with respect to the 140 KDa isoform.

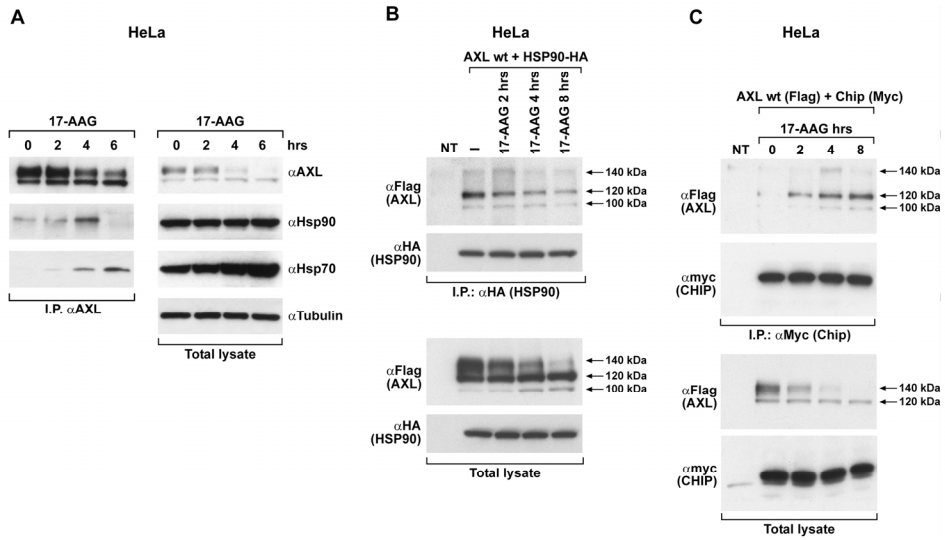


**Figure 9:** (A) HeLa cells treated with 500 nM of 17-AAG for the indicated times were lysed and equal amounts of protein lysate were immunoprecipitated using the anti-AXL antibodies followed by immunoblotting with anti- HSP90 and -HSP70. AXL, HSP90 and HSP70 total levels were monitored using appropriate antibodies in Western blot analysis. Tubulin immunoblotting was used as a loading control. (B) HeLa cells transiently co-transfected with AXL-Flag and HSP90-HA tagged vectors were treated with vehicle (-) or with 500 nM 17-AAG for the indicated times. Equal amounts of protein lysate were immunoprecipitated using the anti-HA antibodies followed by immunoblotting with anti-Flag antibodies. AXL-Flag and HSP90-HA total levels were monitored using the corresponding antibodies in Western blot analysis. NT: non-transfected cells. Arrows indicate the 100, 120 and 140 KDa isoforms of the receptor. (C) HeLa cells co-transfected with AXL-Flag and CHIP-myc were treated or not (-) with 500 nM 17-AAG for the indicated times. Comparable protein aliquots were immunoprecipitated using anti-myc antibody followed by immunoblotting with anti-Flag. AXL-Flag and CHIP-myc total levels were monitored using the anti-TAG antibodies in Western blot. NT indicates non-transfected cells.

### 4.1.4 Results Summary Part I

- The functional blockade of HSP90 by 17-AAG induces AXL receptor tyrosine kinase degradation.
- The fully glycosylated 140KDa AXL isoform is specifically affected by HSP90 inhibition.
- AXL interacts with HSP90 under normal condition and with HSP70 and CHIP E3 ligase upon HSP90 inhibition.
- These observations indicate AXL dependency on HSP90 chaperoning, suggesting AXL as a bona fide HSP90 client.

## 4.2 PART II – Molecular mechanism of 17-AAG induced AXL degradation

### 4.2.1 Proteasomal inhibitors rescue 17-AAG induced AXL Loss

Nearly all proteins in mammalian cells are continually degraded either through the proteasomal or lysosomal machinery and are replaced by *de novo* synthesis. Degradation may also occur as an attempt to eliminate misfolded, damaged or abnormally conformed proteins to counter their accumulation into the cells. Our data clearly demonstrated that AXL is dependent on HSP90 and is degraded presumably due to the induction of misfolding upon HSP90 inhibition; we then assessed the degradation pathway involved in this process. To this aim we used proteasomal inhibitors Lactacystin, MG-132 and the Lysosomal inhibitors Chloroquine or Ammonium chloride along with 17-AAG in CAL62 and HeLa cell lines. Pre-treatment of HeLa and CAL62 cells with proteasomal inhibitors rescued 140 KDa AXL isoform that was depleted upon 17-AAG exposure (Fig.10). Conversely, when cells were treated with lysosomal inhibitors, 17-AAG retained its effect on AXL, indicating that the reduced levels of AXL protein induced by 17-AAG were mediated by proteasomal but not lysosomal degradation machinery.

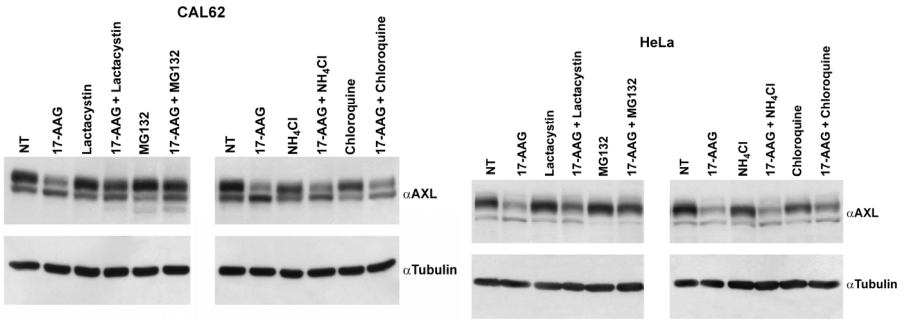


**Figure 10**: Lysates of CAL62 and HeLa cells pre-treated for 1 hr with the proteasomal inhibitors MG132 (10 μM) or lactacystin (5 μM), and the lysosomal inhibitors NH4Cl (20 mM) or chloroquine (100 μM) were treated with vehichle (NT) or with 500 nM of 17-AAG for an additional 3 hrs and immunoblotted for AXL; Tubulin immunoblotting was used as a loading control.

### 4.2.2 17-AAG induced AXL polyubiquitination is mediated by CHIP E3 Ligase

The treatment with 17-AAG by inhibiting HSP90 function alters the composition of the chaperone complex and recruits E3-ubiquitin Ligase, thereby targeting client protein to proteasome for degradation. Proteasomal targeting is often mediated by protein polyubiquitination. To document if AXL undergoes polyubiquitation upon 17-AAG treatment, HeLa cells were transfected with HA-tagged Ubiquitin (ub-HA) and treated with 17-AAG. AXL immunocomplex displayed a time dependent increase in total ubiquitination upon 17-AAG exposure (Fig.11A) with a peak between 2 to 3 hrs of 17-AAG treatment after which ubiquitinated AXL levels decreased. AXL ubiquitination peak preceded the loss of AXL protein at later time points, as shown by the anti-AXL immunoblot. AXL polyubiquitination upon 17-AAG treatment was also verified by immunoprecipitating Ubiquitin (HA) and probing for AXL (data not shown). The treatment with 17-AAG induced progressive accumulation of polyubiquitinated AXL species over time was also confirmed employing Nickel pull-down of His/myc-tagged AXL under denaturing condition; HeLa cells were cotransfected with Ub-HA and His/myc AXL, and purified AXL by Nickel pull down was probed for HA (ub) to evaluate its ubiquitination level (Fig.11B). Since the nickel pull-down was performed under denaturing conditions, this experiment allowed us to exclude that the polyubiquitinated protein observed was AXL itself and not an associated protein pulled down by the AXL immunocomplex, reinforcing that 17-AAG induced AXL-polyubiquitination and targeted it to proteasome degradation machinery.

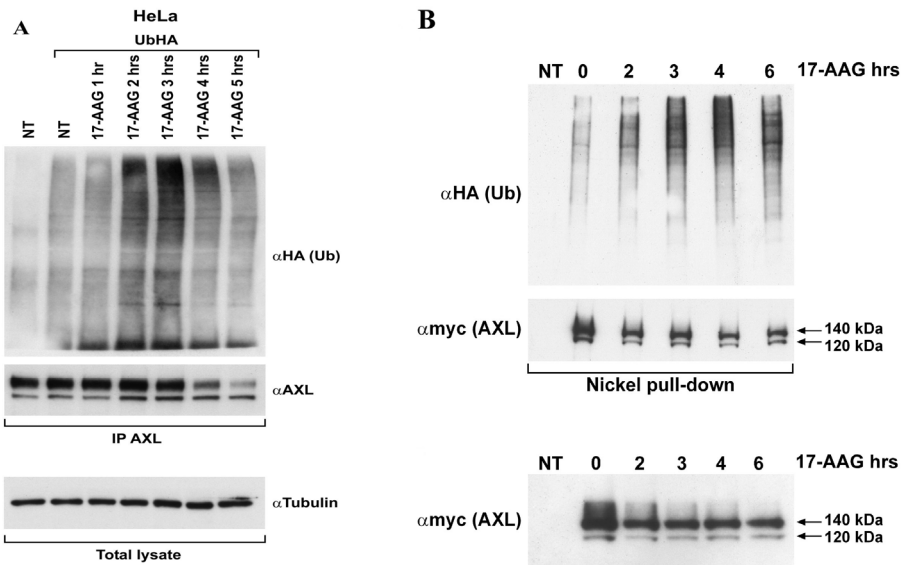

**Figure 11**: (A) HeLa cells, transiently transfected with HA-tagged ubiquitin (ub-HA), were subjected to vehicle (NT) or to 500 nM 17-AAG treatment 48 hrs post-transfection and were harvested at the indicated time points. Upon cells lysis, equal amounts of proteins were immunoprecipitated using anti-AXL antibodies and immunoblotted with antibodies against the HA epitope. AXL immunoblotting is also shown. (B)HeLa cells co-transfected with ub-HA and His tagged AXL (sub-cloned in pCDNA 4 TO A His/myc) were treated or not with 500 nM of 17-AAG for the indicated times. Cells were harvested and comparable amounts of cell suspension were subjected to His-tag (AXL) protein purification under denaturing conditions following the protocol mentioned in the materials and methods section. Nickel affinity purified proteins were resuspended in sample buffer and blotted with anti-HA for Ubiquitin and anti-myc for AXL.

To validate the involvement of CHIP in 17-AAG induced AXL polyubiquitination, we exogenously expressed wt CHIP together with ub-HA in HeLa cells. By immunoprecipitating AXL and probing for HA (ub), we observed an increased AXL polyubiquitination in CHIP wt overexpressing cells. AXL ubiquitination increased in the presence of 17-AAG and was further enhanced by lactacystin treatment, which rescued polyubiquitinated protein from proteasomal degradation (Fig.12A). We also used the CHIP K30A (Xu et al. 2002; Alfano et al. 2010), a mutant in the CHIP TPR domain that acts as a dominant negative mutant since it does not interact with the chaperone complex. When CHIP K30A was ectopically co-expressed with ub-HA in HeLa cells, it was able to abrogate 17-AAG induced AXL polyubiquitination acting in a dominant negative fashion on endogenous CHIP E3 Ligase (Fig. 12A). These data clearly demonstrated the role of endogenous CHIP E3 ligase in 17-AAG induced AXL polyubiquitination (Zhou et al. 2003; Morishima et al. 2008). As expected CHIP wt but not CHIP K30A was capable of interacting with AXL (Fig.12B).

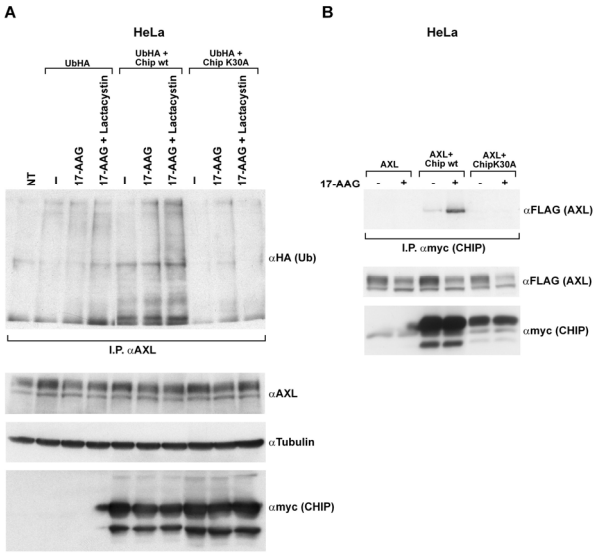


**Figure 12**: (A) HeLa cells co-transfected with Ub-HA and CHIP wt or CHIP K30A was treated with 500 nM 17-AAG (3 hrs) with and without Lactacystin (10 μM) pre-treatment. Cells were lysed and equal amount of proteins were immunoprecipitated using anti-AXL antibodies and immunoblotted with anti-HA antibodies. Anti-AXL immunoblotting was performed to monitor AXL levels upon different treatments and anti-myc immunoblotting to check CHIP expression. Tubulin immunoblotting was used as a loading control. NT: non-transfected cells (B) HeLa cells co-transfected with Flag-tagged AXL (AXL) and myc-tagged CHIP wild type (CHIP wt) or the mutant CHIP K30A, and treated with 500 nM 17-AAG were subjected to immunoprecipitation using anti-myc antibody; CHIP immunocomplexes were probed for anti-Flag antibodies. Anti-Flag and anti-myc immunoblotting of total cell lysates was used to check AXL and CHIP levels, respectively.

### 4.2.3 Effect of 17-AAG on the plasma membrane localized AXL

As we observed that 17-AAG specifically depleted AXL species that was localized on cell surface, we asked whether 17-AAG induced AXL loss was due to a direct effect on the plasma membrane associated receptor. Experimentally, we tested this by biotin pulse-labelling the surface protein in CAL62 cells and subsequently chasing until 6 hrs in the presence of vehicle or 17-AAG. Extracts were pulled-down using streptavidin-conjugated beads to isolate biotin labeled proteins, separated on SDS PAGE and probed for AXL. The outcome of this experiment showed that 17-AAG marginally affected the quantity of surface-bound receptor with respect to vehicle treated cells only at the later time points (Fig.13A), while it strongly affected at its steady state levels at 4-6 hrs of chase, indicating that 17-AAG neither affected the

stabilization of membrane resident receptor nor it accelerated the membrane-localized AXL turnover.

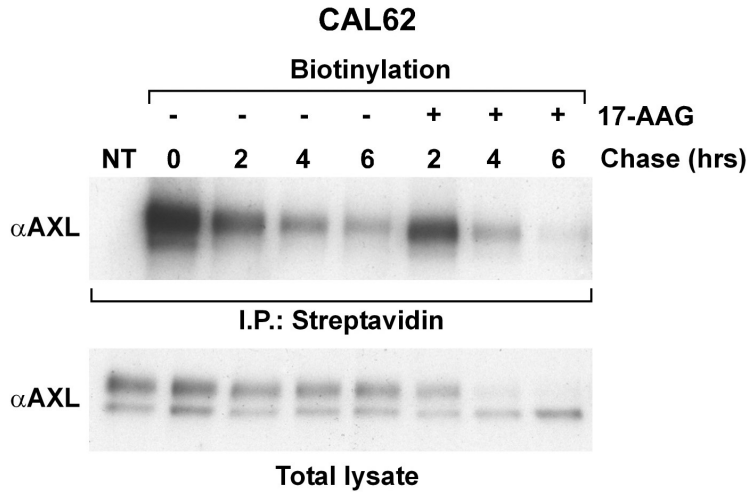


**Figure 13**: CAL62 cells were subjected to 30 min biotinylation of surface proteins and then chased for the indicated times with complete media in the presence or in the absence of 17-AAG (500 nM). Biotinylated proteins were pulled-down using streptavidin-conjugated beads, and the complexes were probed for anti-AXL antibodies on the Western blot. Anti-AXL immunoblotting of total cell lysates was used to check total AXL levels.

To validate the finding that 17-AAG doesn't affect the pre-existing plasma membrane localized AXL receptor, we performed another surface biotin labelling experiment in order to monitor the pre-existing plasma membrane localized AXL isoform (140 KDa) and its fate upon treatment with MG132, 17-AAG or their combination for 5 hrs. This was achieved by biotinylating surface proteins before treatment. Lysates were subjected to Streptavidin agarose pulldown, SDS PAGE and western blotting. As shown in figure 14, MG132 by itself stabilized the pre-existing membrane receptor species, comparing to this no significant improvement of AXL levels were observed when cells were co-treated with 17-AAG and MG132. As expected, 17-AAG treatment did not significantly affect the pre-existing receptor at the membrane.

The same experiment was also performed by biotinylating surface proteins post-treatment and assessing the prevalence of the surface resident AXL, which corresponds to the receptor that is newly synthesized and reached the surface during the treatment course of 5hrs. This revealed that MG132 treatment reduced prevalence of surface receptor in comparison with non-

treated; 17-AAG reduced AXL surface levels and this effect was reverted by MG132 (Fig.14).

These two observations suggested that 17-AAG doesn't affect the surface localized AXL receptor. In contrast, 17-AAG strongly inhibits the delivery of newly synthesized AXL on plasma membrane. Interestingly, MG132 could rescue membrane localised AXL, probably by either blocking AXL proteasomal degradation and/or increasing the half-life of membrane bound receptors as shown in figure 14.

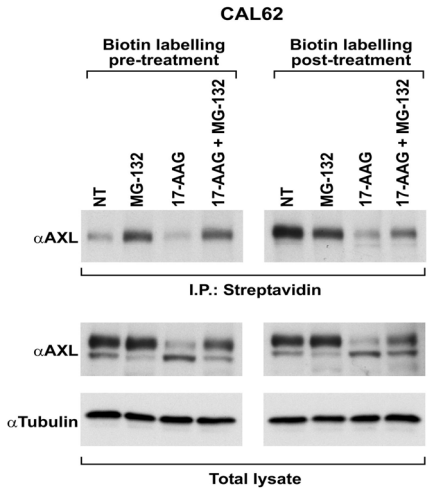


**Figure 14**. CAL62 cells biotinylated before and after treatments with 17-AAG (500nM), MG132 (10μM), and 17-AAG+MG132 for 5hrs, were subjected to streptavidin immunoprecipitation and the recovered biotinylated proteins bound streptavidin agarose beads were denatured, SDS PAGE and western blotting by probing against AXL on the IP streptavidin blots. 5% of the total volume of lysates subjected to immunoprecipitation was used as total lysates for normalization.

### 4.2.4 HSP90 inhibition blocked AXL transport to the cell surface

Having demonstrated that 17-AAG doesn't affect the surface localized species of AXL, we aimed to identify whether HSP90 inhibition affects AXL protein biogenesis/trafficking. To this aim, we metabolically labelled CAL62 cells with [$^{35}$S] cysteine-methionine for 15 min and chased with non-radioactive medium for periods up to 4 hrs in the presence or in the absence of 17-AAG (Fig. 15). In untreated CAL62 cells, AXL completed its glycosylation within 60 min as indicated by the appearance of the 140 KDa isoform. Both 140 and 120 KDa isoforms progressively accumulated, and at 4 hrs of chase,

the 140 KDa AXL isoform is the prevalent one. By contrast, when CAL62 cells were treated with 17-AAG, only the 120 KDa isoform accumulated, and a complete lack of the fully glycosylated receptor was observed. These data suggested that 17-AAG-induced AXL downregulation occurs before reaching the cell surface, and that the drug either impairs maturation of the 120 KDa to 140 KDa AXL isoform and/or induces degradation of 140 KDa isoform in the intracellular component. In either case, 17-AAG blocks the delivery of fully glycosylated AXL protein to cell surface.

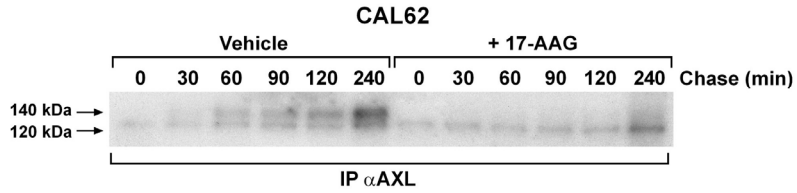


**Figure 15**: CAL62 cells grown in complete medium were exposed to cysteine-methionine-free DMEM containing dialyzed 2% FBS for 1 hr. Cells were then pulsed with $^{35}S$ methionine/cysteine for 15 minutes and chased with nonradioactive medium for the indicated times with or without 17-AAG (500 nM). At each time point, cells were lysed and subjected to immunoprecipitation with anti-AXL antibodies, SDS PAGE and Gel dried was exposed to radioactivity.

### 4.2.5 Kinase domain but not the kinase activity sensitises AXL to 17-AAG

To localize the region that sensitizes AXL to 17-AAG, we used the AXL-EC mutant that lacks the entire intracellular domain and has only the extracellular and transmembrane domain. As a control we used a similar construct of the HER2 receptor (HER2-EC), because HER2 is a well established HSP90 client. It has been shown that HER2 sensitivity to 17-AAG requires its kinase domain. Moreover, both the immature and completely glycosylated isoforms of HER2 are equally sensitive to 17-AAG, differently from what we observed in AXL. In addition to the extracellular alone mutants we also used AXL/HER2, which is a chimera of AXL extracellular domain fused to HER2 tyrosine kinase domain, and the reverse chimera where the tyrosine kinase domain of AXL is fused to extracellular domain of HER2 ie., HER2/AXL. We transiently transfected these constructs in HeLa cells and tested their sensitivity to 17-AAG. The result showed that the AXL-EC and HER2-EC are insensitive to 17-AAG (Fig.16B), depicting that similar to HER2 it is the kinase domain of AXL that offers sensitivity to 17-AAG. In addition to the EC mutants, we also used AXL/HER2, which is a chimera of AXL

extracellular domain fused to HER2 tyrosine kinase domain, and the reverse chimera where the tyrosine kinase domain of AXL is fused to extracellular domain of HER2 ie., HER2/AXL. As shown in Figure 16B, 17-AAG sensitivity was retained when the two kinase domain were swopped between the two receptors.

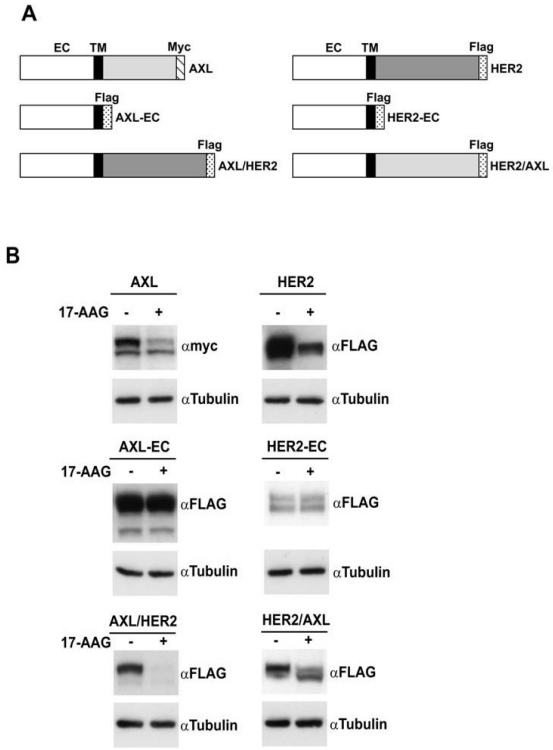


**Figure 16**: (A) Schematic representation of AXL wt, HER2 wt, AXL-EC, HER2-EC, AXL/HER2 and HER2/AXL (EC-Extracellular domain, TM-Transmembrane domain, shaded portion-Tyrosine kinase domain). (B) HeLa cells were transiently transfected with wild type, EC mutants and chimeric constructs of AXL and HER2 expression vectors and treated with 500 nM 17-AAG (+) or vehicle (-) for 8 hrs and the lysates were immunoblotted with anti-Flag/myc. Tubulin immunoblotting was used as a loading control.

Having shown that the kinase domain of AXL confers sensitivity to 17-AAG, we then asked whether the kinase activity is also required. To this aim, we used CAL62 cells that presents phosphorylated AXL due to intrinsic presents of its ligand Gas6 (Avilla et al. 2011). We also found, Bosutinib that is reported to abrogate AXL phosphorylation (Asish et al., 2011) do interfered with AXL kinase activity in this system. We then tested Bosutinib pre-treated CAL62 cells for 17-AAG induced degradation of AXL. This experiment revealed no change in the degradation kinetics between 17-AAG treated and

17-AAG and bosutinib co-treated cells (Fig.17A). Consistent with this, the AXL kinase dead mutant (AXL K558R) overexpressed in HeLa cells retained its sensitivity to 17-AAG confirming that the kinase activity is not necessary for 17-AAG induced AXL degradation (Fig.17B).

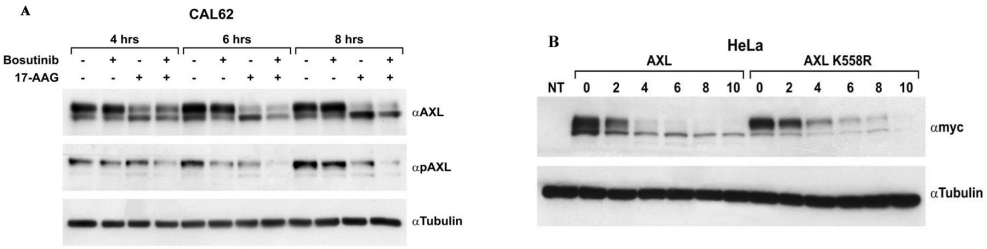


**Figure 17:** (A) CAL62 cells pretreated with Bosutinib (5μM) were co-treated or not with 17-AAG, harvested at the indicated time points and lysates were subjected to western Blotting. Anti-AXL, anti-phospho AXL and Tubulin were probed to assess the degradation kinetics, phosphorylation status and normalization respectively. (B) HeLa cells transfected with AXL WT and AXL KD were assessed for its 17-AAG sensitivity by treating them with 500 nM 17-AAG and harvested on time course. The lysates were subjected to western blotting and probed against their respective tag. Tubulin immunoblot was performed for normalization.

### 4.2.6 Results Summary Part II

- The proteasomal inhibitors MG132 and Lactacystin recovered the 17-AAG induced AXL loss of 140 KDa AXL isoform suggesting the involvement of proteasomal machinery in this process.
- The intervention of proteasomal machinery is confirmed as AXL displayed a progressive accumulation of its polyubiquitinated species upon 17-AAG preceding receptor degradation.
- Like most other HSP90 clients, it is the CHIP E3 ligase that mediates AXL polyubiquitination upon induction of its misfolding by HSP90 inhibition.
- In contrast to other HSP90 clients, the specific effect of 17-AAG on AXL 140 KDa isoform is probably due to the degradation of trafficking AXL in the cytosol, rather than directly targeting the membrane localised fully glycosylated AXL on the plasma membrane.
- It is the AXL Kinase domain that confers sensitivity of this receptor to 17-AAG.
- The kinase activity of the receptor doesn't play a role in 17-AAG sensitivity.

## 4.3 PART III – The effect of 17-AAG on AXL driven signalling and biological activity

### 4.3.1 17-AAG targeting AXL interferes with its signalling and biological activity

Having demonstrated the effect of HSP90 inhibition over AXL, we then intend to address the impact of 17-AAG on AXL-driven signalling and biological activity. To this aim, we used two different ATC cell lines: CAL62 and 8505C, previously characterized for their dependence on AXL signalling in acquiring survival advantage. As expected 17-AAG exposure of these cells induced a reduced AXL phosphorylation concomitant with its decreased total protein. In addition, upon 17-AAG treatment we observed the reduced activation of AKT and p70-S6 kinase (Fig.18A) upon 17-AAG, which are the predicted AXL downstream substrates in these models (Avilla et al. 2011). As a control, we could show similar effects over AXL phosphorylation and signaling using the AXL kinase inhibitor Bosutinib. Moreover, as a readout of AXL-induced biological activity, we used an AP-1 dependent Luc vector. The transfection of the AP1 Luc vector in HeLa cells did not result in significant Luc activity. Instead, AXL co-expression induced a strong activation of AP-1 dependent promoter activity. Such activity was inhibited by 17-AAG in a dose-dependent fashion, with a reduction of approximately 50% at 100 nM, and complete inhibition of promoter activation at 200 nM (Fig.18B). This data indicated that 17-AAG exposure was able to selectively interfere with AXL mediated biological activity.

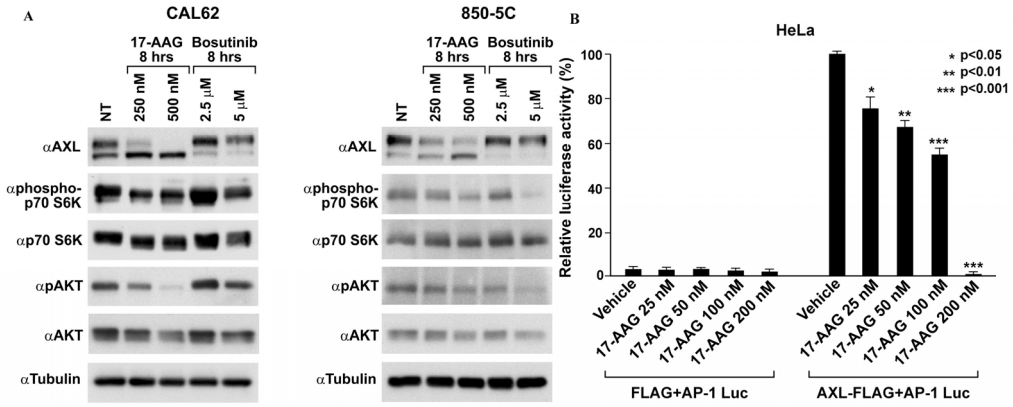


**Figure 18**: (A) CAL62 and 8505C cells treated with 17-AAG and Bosutinib in varying doses were subjected to western blotting. The signalling substrates of AXL in these models like p70S6 Kinase and AKT was probed with the respective phospho and total antibodies. Tubulin

immunoblotting was used as a loading control. (B) HeLa cells were transiently transfected with AXL-Flag expressing vector and the AP1-Luc vector. pRL-null (a plasmid expressing the enzyme Renilla luciferase from Renilla reniformis) was used as an internal control. Firefly and Renilla luciferase activities are expressed as percentage of residual activity of 17-AAG treated cells with respect to untreated cells. Average results of three independent assays ± S.D. are indicated. The ANOVA Bonferroni multiple comparison test was used to demonstrate statistical significance.

### 4.3.2 Results Summary Part III

- 17-AAG induced AXL loss resulted in a reduced AXL phosphorylation and signalling.
- The HSP90 inhibition selectively interfered with AXL mediated biological activity.

# 4. DISCUSSION

AXL overexpression and activation has been implicated in the progression of several human cancers and in mediating drug resistance. In addition, we recently reported the overexpression and activation of AXL in thyroid carcinoma. We demonstrated the crucial role of AXL in sustaining proliferation, survival, motility and invasiveness in driving thyroid carcinoma, supporting the notion that AXL could be oncogenic when overexpressed and hyperactive. Despite the number of evidences showing the oncogenicity of AXL, the advent of specific and clinically relevant antagonists like antibodies or tyrosine kinase inhibitors against AXL remained elusive. These antagonistic approaches against AXL continued to be challenging due to the lack of evidence illustrating the regulation of AXL over-expression and its innate capability to avail activation through diverse modes. For instance, AXL can be activated by (i) ligand dependent dimerization, (ii) ligand independent dimerization, (iii) heteromeric dimerization with the other TAM family receptors, (iv) heterotypic dimerization with a non-TAM Family receptor, and (v) trans-cellular binding of extracellular domains (Hafizi et al. 2006 & Linger et al. 2011). According to published preclinical reports the kinase inhibitors MP470, SKI-606 (Bosutinib) and R428 showed inhibitory effect on AXL signalling along with other targets. In addition, a phage-derived mAb (YW327.6S2) that recognizes both human and murine AXL (Ye et al. 2010) displayed antagonistic effect on Gas6 mediated AXL signalling. However, the specificity and the convincing effect of these agents against the complex AXL signalling at the clinical setting still represent major concerns that have not yet been addressed.

We attempted to provide evidence that AXL could be effectively targeted employing a clinically relevant HSP90 inhibitor, Ansamycin antibiotic Geldanamycin analog 17-AAG. The usage of Ansamycin antibiotics to downregulate RTKs represents a promising tool in deactivating HSP90 dependent oncogenic signal transducers. Based on the unique and therapeutically attractive effect of HSP90 in regulating multiple tumor survival and progression pathways, extensive studies in developing HSP90 inhibitors are being performed, and the number of inhibitors is rapidly increasing. More importantly, studies have shown that 17-AAG binds to HSP90 in cancer cells with about 100-fold higher affinity than it does in normal cells (Kamal et al. 2003). The difference might come from the different states of HSP90 in cancer cells and normal cells because the tumor HSP90 is mostly in multi-protein complexes with high ATPase activity, probably due to the increased load of

mutant client proteins (Kamal et al. 2003); whereas, HSP90 from normal cells are mainly in an uncomplexed state that has low ATPase activity (Kamal et al. 2003).

HSP90 and its related co-chaperones play a regulatory role in maintaining conformational maturation and structural integrity of a variety of cellular proteins. In this study we show that AXL is dependent on HSP90 for its maturation and continued transport to the plasma membrane. Employing 17-AAG, we observed that AXL is degraded in a panel of thyroid carcinoma cell lines as well as in HeLa cells. This effect was reproduced when a chemically unrelated drug, Radicicol was used to inhibit HSP90. Interestingly, AXL degradation resulting from 17-AAG affected disproportionately the completely glycosylated receptor (140 KDa), rather than the newly synthesized (100 KDa) or the partially glycosylated AXL species (120 KDa). Hence, the exposure to 17-AAG reduced the prevalence of AXL receptor on the plasma membrane. This specific effect of depleting only the fully matured receptor was observed for AXL but not in other HSP90 clients c-KIT, RET and PDGFR that are sensitive at all stages of maturation.

The effect of 17-AAG on AXL protein was clearly shown to have functional relevance to HSP90 inhibition, as we could show the interaction between HSP90 and AXL under normal conditions; and it was curtained upon 17-AAG exposure on time. Besides this, the AXL immunocomplex displayed an increasing association to HSP70 on time with 17-AAG. This shift in the associating partners of chaperone complex with AXL, not only demonstrates the alteration in the multichaperone complex but also indicates the fate of AXL protein within the complex. The shift from the normal folding mode to the degradation mode of chaperone complex, mediates CHIP E3 ligase recruitment and links the chaperone associated client to proteasome degradation machinery (Kundrat and and Regan 2010).

CHIP E3 ligase recruitment in the AXL associated chaperone complex observed only upon HSP90 inhibition. The ability of wt CHIP, but not of CHIP TPR mutant (CHIP K30A), to ubiquitinate AXL supports the involvement of CHIP E3 ligase in AXL-polyubiquitination at least in the context of HSP90 inhibition. Though CHIP K30A was capable of abolishing AXL ubiquitination, it could not completely stop the loss of AXL induced by 17-AAG. These data were also confirmed by RNA interference of CHIP in HeLa and CAL62 cells (data not shown). We cannot exclude that our approaches could not achieve a complete inhibition of CHIP function, and the residual activity might be still sufficient to degrade client proteins. However, the possible involvement of other E3 ligases in 17-AAG induced protein degradation of HSP90 clients

should be considered as well. By using CHIP$^{-/-}$ mouse embryo fibroblasts, it has been shown that CHIP role in ubiquitination and turnover of HSP90 client proteins is not exclusive and that other E3 ligases may complement CHIP deficiency (Xu et al. 2002; Morishima et al. 2008). Moreover, our data also suggest the possibility of an alternate pathway that guides the clearance of the misfolded proteins.

Though the concept of client RTK destabilization upon HSP90 inhibition came into light decades ago, this is the first report dissecting the HSP90 inhibitory effect on differentially glycosylated species of a client protein. The molecular explanation behind this differential effect was obtained by analysing the steady-state AXL levels and surface receptor turnover, by assessing the MG132 rescued AXL species from 17-AAG induced degradation and by tracking the newly synthesized and the trafficking AXL in the presence and in the absence of 17-AAG.

Our analysis on steady state level showed that AXL loss induced by 17-AAG is at the plasma membrane, and the proteasomal inhibitor MG132 did rescue this 17-AAG induced effect. Despite, neither HSP90 nor CHIP E3 ligase immunocomplexes showed a predominant association with the membrane associated 140 KDa AXL species but interaction observed significantly with 120 and 100 KDa AXL isoforms. This insignificant association of 140 KDa species to HSP90/CHIP is due to the phosphorylation gained in that isoform rendering the interaction more fragile and hence detection was improbable; this we confirmed using the AXL kinase dead mutant that displayed a predominant association of its 140KDa isoform likewise 120 and 100 KDa isoform to HSP90/CHIP immunocomplexes (data not shown). The surface protein biotin labelling to assess the turn-over of membrane localized AXL showed that the destiny of the pre-existing membrane localized AXL remained unaffected, at least until 4hrs of chase points in the presence of 17-AAG.

Surface protein biotin labelling followed by MG132 treatment alone or in combination with 17-AAG aided to monitor the fate of the pre-existing membrane localized AXL along with the identity of the rescued species by MG132. This experiment showed that MG132 stabilized the pre-existing surface localized receptor, while 17-AAG did not affect AXL levels. The specific effect of MG132 in stabilizing the surface localized AXL isoform could partially explain AXL 140 KDa recovery observed in western blotting analysis of total proteins under co-treatment conditions (Fig.14B), however, we cannot exclude that part of this MG132 recovery is also be due to the rescued species from 17-AAG effect, since on total lysates MG132 barely increases

AXL levels compared to non-treated but does a significant recovery on co-treatment with 17-AAG. The fate of newly synthesized membrane localized AXL in the presence of 17-AAG and MG132 has been assessed by biotin labelling after treatments with the two compounds. MG132 reduced AXL prevalence on cell surface, and this probably due to overload of secretary pathway with polyubiquitinated proteins. 17-AAG treatment strongly reduced AXL appearance on the cell surface, and MG132 co-treatment reverted this effect. However, this reversion might include the stabilized pre-existing surface resident AXL as well as the recovered AXL transport to the cell membrane that bypassed the 17-AAG effect. Whatever the case, our data clearly emphasized that 17-AAG does not affect plasma membrane localized AXL. Furthermore, by exploiting $^{35}S$ methionine pulse-chase experiment, we observed no 140 KDa species in the presence of 17-AAG, by contrast, in untreated cells AXL accumulated first as 120 KDa isoform and then as a 140 KDa species that represents the mature membrane exposed receptor. This observation suggests 17-AAG depletes the completely matured AXL species by blocking its maturation and degrading the receptor at the cytosol before having reached the membrane.

Other HSP90 client proteins, such as the HER2, RET, PDGF or c-KIT receptor tyrosine-kinases require chaperone activity for the correct folding and stability of both mature and immature forms. In contrast to HER2, the TrkAI receptor is similar to AXL, because it requires HSP90 for its maturation and cell surface translocation. In fact, 17-AAG treatment of TrkAI expressing cells causes the degradation of the fully mature receptor and the accumulation of the immature one (Farina et al. 2009).

Our data also showed that an AXL receptor carrying an intact tyrosine kinase domain retained sensitivity to 17-AAG, while the AXL-EC, an ICD deletion mutant completely lost the response. Moreover, when the tyrosine kinase domains of AXL and HER2 were swopped between each other, both the chimeric constructs retained the sensitivity to 17-AAG, despite their ICD deletion mutants (AXL-EC and HER2-EC) failed to respond. This reinforces the hypothesis that like HER2, it is the TK domain of AXL that is necessary to avail sensitivity towards HSP90 inhibitors. Furthermore, our data proved that AXL kinase activity is not required for this effect. This is in favour of the hypothesis that the HSP90 binding determinants are located in the kinase domain, and this association depends on the intrinsic structure and stability of the receptor TK domain (Shimamura et al. 2005; Taipale et al. 2012) rather than on its activity. AXL overexpression and activation appears to be a feature of many different types of cancer and there is enough evidence to support its

oncogenic activity (Li et al. 2009). We have previously demonstrated that AXL is overexpressed and active in thyroid cancer. In thyroid carcinomas, AXL ligand, GAS6, is also frequently expressed either by thyroid cancer cells or, in some cases, by stromal cells. We have shown that silencing AXL expression by RNA interference hampers thyroid cancer cell proliferation, survival, migration and tumor formation in immunodeficient mice (Avilla et al. 2011). Moreover, it has been reported that AXL overexpression is a frequent mechanism of resistance to different anticancer agents (Huang et al. 2010; Liu et al. 2009; Hong et al. 2008; Dufies et al. 2011; Zhang et al. 2012). These documented evidences clearly put forth the role of AXL in pathogenesis and progression of various human malignancies. We have shown that 17-AAG inhibits AXL activation, signalling and biological activity. However, we cannot exclude that such effects could likely be due to the cumulative effect of 17-AAG on several HSP90 client proteins, rather than AXL alone.

# 6. CONCLUSION

The molecular chaperone HSP90 is a promising target of anti-cancer drugs. Many of the HSP90 "client" proteins are involved in cancer signaling pathways. Hence, inhibition of HSP90 is predicted to simultaneously affect multiple oncogenic signaling pathways. Several HSP90 inhibitors, including 17-AAG, show very high selectivity for HSP90 in tumor cells compared to normal cells. 17-AAG has entered phase I/II clinical trials with very good effectiveness. Many other inhibitors are under preclinical development. Several preclinical and clinical reports have illustrated that chemical optimization of HSP90 inhibitors with improved efficacy and selectivity might be effective against therapeutically complex tumors.

Our attempt to validate and address the effects and mechanism of 17-AAG on AXL downregulation indicates that AXL as a bona fide client of HSP90 and the therapeutic approach to target HSP90 would counteract AXL overexpression and its aberrant activation in human malignancies along with the growing list of HSP90 oncogenic clients. Besides providing evidence that AXL can be targeted via HSP90 inhibition, our data also widens the understanding of HSP90 chaperoning, its biological importance and more importantly the therapeutic modality of using HSP90 inhibitors as an anti-cancer agent at least against selective or tumor addictive HSP90 clients.

Our data convincingly demonstrated that AXL can be listed among HSP90 clients. In the advent of optimized HSP90 inhibitors, we strongly recommend AXL addicted tumors or drug resistance conditions may be considered as an optimal scenario to employ HSP90 inhibitors to counteract AXL.

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# APPENDIX

Cancer Research

# Activation of TYRO3/AXL Tyrosine Kinase Receptors in Thyroid Cancer

Elvira Avilla, Valentina Guarino, Carla Visciano, et al.

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# Activation of TYRO3/AXL Tyrosine Kinase Receptors in Thyroid Cancer

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## Abstract

Thyroid cancer is the most common endocrine cancer, but its key oncogenic drivers remain undefined. In this study we identified the *TYRO3* and *AXL* receptor tyrosine kinases as transcriptional targets of the chemokine CXCL12/SDF-1 in CXCR4-expressing thyroid cancer cells. Both receptors were constitutively expressed in thyroid cancer cell lines but not normal thyroid cells. AXL displayed high levels of tyrosine phosphorylation in most cancer cell lines due to constitutive expression of its ligand GAS6. In human thyroid carcinoma specimens, but not in normal thyroid tissues, AXL and GAS6 were often coexpressed. In cell lines expressing both receptors and ligand, blocking each receptor or ligand dramatically affected cell viability and decreased resistance to apoptotic stimuli. Stimulation of GAS6-negative cancer cells with GAS6 increased their proliferation and survival. Similarly, siRNA-mediated silencing of AXL inhibited cancer cell viability, invasiveness, and growth of tumor xenografts in nude mice. Our findings suggest that a TYRO3/AXL-GAS6 autocrine circuit sustains the malignant features of thyroid cancer cells and that targeting the circuit could offer a novel therapeutic approach in this cancer. *Cancer Res; 71(5); 1792–804. ©2011 AACR.*

## Introduction

Thyroid cancer is the most common endocrine malignancy and its incidence is increasing worldwide. Thyroid cancer histotypes include: well-differentiated papillary and follicular (PTC and FTC), poorly differentiated (PDTC) and anaplastic thyroid carcinoma (ATC; ref. 1, 2, 3). Thyroid cancer features overexpression of specific chemokine and their receptors (4, 5). CXCR4/SDF-1 axis has an important role in promoting cell growth, invasiveness, and survival in thyroid cancer cells and its blockade can revert all these phenotypes (4, 5, 6). We analyzed global gene expression profiles of CXCR4-expressing human PTC cells (TPC-1) with or without SDF-1α. We identified *TYRO3* and *AXL*, belonging to the TAM family (Tyro3, Axl, and Mer) of tyrosine kinase receptors (7, 8). TAM-mediated signaling is involved in cell survival, proliferation, migration and adhesion (9), vascular smooth muscle homeostasis (10), platelet function (11; 12), and erythropoiesis (11). These receptors are frequently coexpressed in vascular, reproductive, nervous, and immune system in adults (13). TAM receptors can be activated by 2 physiological ligands, GAS6 (Growth-Arrest-Specific gene 6) and Protein S, which are homologous vitamin-K–dependent proteins (13). TAM receptors are involved in cancer development and progression. AXL overexpression is observed in many human cancer types. Gas6 is frequently expressed in cancer and its level correlates with poor prognosis (14).

The aim of this report is to study TAM receptor involvement in thyroid cancer. We show that human PTC/ATC cells and samples, but not normal thyroid, constitutively express AXL, TYRO3, and their ligand GAS6. We show that AXL, TYRO3, and GAS6 have a critical role in mediating thyroid cancer cell proliferation, invasiveness, and survival. Moreover, we show that silencing of AXL in an ATC cell line strongly affects tumor growth in immunodeficient mice. These data provide evidence that the AXL/TYRO3/GAS6 axis might be exploited as target for potential antitumoral therapy.

## Materials and Methods

### Cell cultures

Human primary cultures of normal thyroid were obtained from F. Curcio (Dipartimento di Patologia e Medicina Sperimentale e Clinica, University of Udine, Italy;) and cultured as described (15). Human thyroid papillary cancer cell lines TPC1, BCPAP, NIM, and anaplastic thyroid cancer cell lines 850–5C, CAL62, U-HTH83, C643, SW1736, U-HTH7, U-HTH83, C643, SW1736, OCUT-1, ACT-1 have been previously described (15–17). PC, PC BRAF, PC HaPTC3, PC E1A, PC PTC1, PC E1A-RAF, PC PTC3, PC v-mos, PC E1A-RAF rat thyroid epithelial cells have been previously described (4, 5, 18). Transformed human embryonic kidney cells (293T) have been obtained by ATCC (19). TPC1 and NIM were obtained from Dr. M. Santoro

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(University of Naples, Naples, Italy); BCPAP, CAL62, and 850-5C were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. OCUT-1 and ACT-1 were a gift of K. Hirakawa and N. Onoda (Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka, Japan) and SW1736, C643, U-HTH7, and U-HTH83 cells were a gift of C.H. Heldin (Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden). All cells have been tested for specific mutations and thyroid marker expression. Cells are passaged for fewer than 3 months.

### Histology and immunohistochemistry

Thyroid tissue samples from patients affected by thyroid carcinomas and normal thyroid tissues were obtained from the Struttura Complessa di Anatomia Patologica, Istituto Nazionale Tumori of Naples, upon informed consent. Anti-AXL, anti-GAS6, and anti-CXCR4 antibodies from R&D Systems were used to stain human thyroid carcinoma samples. For xenograft hystological analysis, Ki67 antibody (Clone MIB-1) was from Dako, and CD31 antibody was from Santa Cruz Biotechnology; samples were processed with peroxidase detection system reagent kit (Novocastra). Apoptosis was evaluated by the terminal deoxynucleotidyl transferase (TdT)-mediated dNTP-labeling (TUNEL) method using Fragel DNA fragmentation detection kit colorimetric-TdT enzyme by Calbiochem—Merck KgaA (Darmstadt). Four μm-thick sections were deparaffinized and rehydrated and antigen retrieve technique was carried out in pH 6.0 buffer in a microwave for 3 minutes using standard histological technique. Evaluation has been done by an expert pathologist (R. Franco). For vessel examination, the stained sections were screened at low power (×40), and 3 areas with the most intense neovascularization (hot spots) were selected. Mean vessel lumen cross-sectional diameter were determined for 10 hot spot ×40 areas, all counted based upon spatial calibration parameters established with a slide digital micrometer (Olympus cellSens digital image analysis system).

### RNA interference

*SMART*pools (custom-synthesized siRNA) by Dharmacon were used for *AXL*, *TYRO3*, and *GAS6* silencing. Cells were grown under standard conditions. The day before transfection, cells were plated in 6-well dishes at the density of $3 \times 10^5$. Transfection was done by using 100nmol/L of *SMART*-pool and 6 μL of Dharma*FECT* (Dharmacon). Cells were harvested at 24 hours after transfection and analyzed by WB and FACS.

### Generation of stable *AXL* shRNA- and *AXL* dominant-negative expressing cell lines

We obtained 5 lentiviral constructs (pLKO.1*puro*) containing 21-mer short hairpin RNAs (shRNA) directed to various coding regions of *AXL* (Mission shRNA, pLKO.1 *puro*) from Sigma-Aldrich, Inc (20). 850–5C were electroporated with the pool of shRNAs directed against *AXL* or with a pool of nontargeting vectors (shCTR). Stable transfectants were selected in medium with 500ng/mL puromycin. To block AXL, we also generated 850-5C cells that stably express dominant-negative forms of the receptor, that is FLAG-tagged *AXL* EC, that lacks the whole intracellular domain, and the kinase dead mutant of *AXL* carrying the mutation K558R, which abrogates TK activity (Myc-tagged AXL KD). Detailed experimental procedures regarding these mutants are included in Supplementary data.

### Protein studies

Protein extractions, immunoblotting, immunoprecipitation, and pull-down binding assays were carried out according to standard procedures. Anti-AXL were from Santa Cruz Biotechnology. Anti-TYRO3 was from R&D Sysyems. Anti-phospho AXL, specific for tyrosine 779, was from R&D Systems. Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology. Anti-AKT and anti-phospho-AKT were from Cell Signaling. Anti-phospho-p44/42 MAPK and anti-p44/42 MAPK were from Cell Signaling. Anti-phospho-p70S6K and anti-p70S6K were from Cell Signaling. Monoclonal anti-tubulin was from Sigma Chemical Co. Anti-GRB2 antibodies were from Santa Cruz Biotechnology; anti-p85 was from Cell Signaling. Secondary anti-mouse, anti-goat, and anti-rabbit antibodies coupled to horseradish peroxidase were from Bio-Rad. Detailed experimental procedures can be found in Supplementary data.

### ELISA assay

Thyroid cells were allowed to grow 70% confluency and serum-deprived for 12 hours. GAS6 levels in culture supernatants were measured using a quantitative immunoassay ELISA kit (DuoSet ELISA Development Kit, R&D Systems). Samples in triplicate were analyzed at 450 nm with an ELISA reader (Model 550 microplate reader, Bio-Rad).

### Matrigel invasion

*In vitro* invasiveness through Matrigel was assayed using transwell cell culture chambers according to described procedures (4). Cells ($1 \times 10^5$ cells/well) were added to the upper chamber of a prehydrated polycarbonate membrane filter of 8 μmol/L pore size (Costar, Cambridge) coated with Matrigel (Collaborative Research) and then incubated at 37°C in a humidified incubator in 5% CO2 and 95% air for 24 hours. Migrating cells on the reverse side of the filter were stained with 0.1% crystal violet in 20% methanol for 30 minutes, lysed in 10% acetic acid, and analyzed at 570 nmol/L with an ELISA reader (Model 550 microplate reader, Bio-Rad). The results were expressed as percentage of migrating cells with respect to the sh-CTR silenced ones.

### Cell proliferation

S-phase entry was evaluated by 5-bromo-2-deoxyuridine (BrdU) incorporation and indirect immunofluorescence according to described procedures (4, 5). Cells were grown on coverslips, serum starved for 12 hours, and treated with recombinant human GAS6 (200ng/mL) for 24 hours. Moreover, cells were silenced with siRNA (Dharmacon) for 24 hours, serum-deprived for 12 hours, and then treated with recombinant human GAS6 (200ng/mL) for 24 hours. BrdU was

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added at a concentration of 10 μmol/L and BrdU-positive cells were revealed with Texas-Red–conjugated secondary antibodies (Jackson Immuno Research Laboratories).

For growth curves, cells were plated at a density of $0.5 \times 10^5$ in low serum conditions (2.5%) and counted at the indicated time-points.

### Flow cytometric analysis (FACS)

Cells were transfected with control siRNA (siCTR) or AXL-targeting siRNAs (siAXL) and 48 hours later detached from culture dishes with a solution of 0.5 mmol/L EDTA. After saturation with 1 μg of human IgG/$10^5$ cells, cells were incubated for 20 minutes on ice with antibodies against AXL extracellular domain (R&D Systems) or isotype control antibody. Unreacted antibody was removed and cells were incubated with phycoerytrin-labeled secondary antibodies (R&D Systems). Cells resuspended in PBS were analyzed on a FACS-Calibur cytofluorimeter using the CellQuest software (Becton Dickinson). Analyses were done in triplicate. In each analysis, a total of $10^4$ events were calculated.

### TUNEL assay

For TUNEL, an equal number ($5 \times 10^3$) of cells was seeded onto single-well Costar glass slides; after 24 hours of silencing, cells were serum-deprived for 12 hours and treated with GAS6 (200 ng/mL) for 24 hours, and subjected to the TUNEL reaction (Roche) as described elsewhere (4).

### Xenografts in nude mice

Each group of 10 mice (4-week-old male BALB/c nu/nu mice, Jackson Laboratories) was inoculated subcutaneously into the right dorsal portion with 850-5C shCTR cells, 850-5C shAXL Cl2, 850-5C shAXL CL4, or 850-5C shAXL CL6 ($10 \times 10^6$/mouse). Tumor diameters were measured at regular intervals with the caliper. Tumor volumes (V) were calculated with the formula: $V = A \times B^2/2$ (A = axial diameter; B = rotational diameter). This study was conducted in accordance with Italian regulations for experimentation on animals.

### Statistical analysis

For comparisons between cell lines, we used the parametric 2-tailed *t*-test. To calculate the association between CXCR4 and AXL expression in PTCs, we used the 2-sided Fisher's Exact Test, with the GraphPad Instat software, v.3.0b. Differences were statistically significant at $P < 0.05$.

## Results

### TYRO3 and AXL receptors are overexpressed and activated in thyroid cancer cells

We carried out global gene expression profiling of human TPC1 cells with or without SDF-1α. Among several genes, *TYRO3* and *AXL* were significantly upregulated. Quantitative RT-PCR on selected genes and Western blot analysis on TYRO3 and AXL confirmed these findings (Supplementary Fig. S1A and B). Treatment of thyroid cancer cell lines with the CXCR4 inhibitor AMD3100 inhibited SDF-1-mediated AXL upregulation (data not shown), but not constitutive AXL expression, indicating that other mechanisms are responsible for this phenomenon. Thyroid cancer cell lines carry activated oncogenes belonging to the *RAS-BRAF-ERK*, the *p53* or the *PI3K/AKT* pathway. TYRO3 and AXL protein levels were undetectable in normal thyroid cells, whereas they were significantly expressed in cancer cell lines (Fig. 1A). This was also confirmed by RT-PCR experiments (Supplementary Fig. S1C). AXL is activated in different cancer histotypes (21–25). Thus, we evaluated its tyrosine-phosphorylation levels in our cells by using both anti-phosphotyrosine and phospho-Tyr 779 (Y779) antibodies. Cancer cells displayed different degree of AXL tyrosine-phosphorylation (Fig. 1A). TPC1 cells, derived from a human PTC featuring a spontaneous RET/PTC1 rearrangement, showed the highest levels. Many thyroid cancer cell lines (850-5C, NIM, and CAL62) expressed the AXL ligand GAS6, but not TPC1 (Fig. 1B and Supplementary Fig. S1C). AXL was also overexpressed in most of the oncogene-transfected rat thyroid cells lines (PC Cl3) but not in PC E1A and PC BRAF (Fig. 1C). Again, PC PTC cells show the highest level of AXL phosphorylation (Fig. 1C). These data suggest that RET/PTC may be involved in AXL activation through a ligand-independent mechanism, whereas other genetic lesions induce a ligand-dependent activation of the receptor. AXL, through a C-terminal multidocking tyrosine (Y821), binds to several substrates, such as p85 and GRB2. Tyrosine 779 was identified as an additional p85-binding site (26). Although p85 activation invariably results in the activation of the phosphatidylinositole 3-kinase (PI3-K)/Akt pathway, GRB2 can signal to both the PI3-K/Akt and the extracellular regulatory kinase (ERK) pathways (27). We analyzed the pattern of AXL-binding proteins in TPC-1 and 850–5C cells by AXL immunoprecipitation followed by immunoblotting with anti-phosphotyrosine antibodies. p85, but not GRB2 (not shown), coprecipitated with AXL (Fig. 1D). However, in p85 immunoprecipitates, we could not detect AXL. This could be due to the interference of p85 antibody with AXL/p85 interaction, or to the limiting amount of endogenous proteins in our system. To distinguish between these possibilities, we overexpressed AXL and p85 in 293T cells and showed the presence of AXL in p85 immunoprecipitates and in GST-SH2 p85 pull-down assays (Supplementary Fig. S2). By using TPC1 and 8505-C cell lysates, AXL binding was detected in N-terminal p85 SH2- (Supplementary Fig. S3), but not GRB2 SH2 (data not shown) pull-down experiments. Consistently, the phosphorylation of AKT, but not of ERK1/2, was observed upon GAS6 stimulation of thyroid cancer cells (Supplementary Fig. S3). We also overexpressed AXL and GRB2 in 293T cells. In this experiment, we detected AXL-GRB2 coimmunoprecipitation, but GRB2 binding was not observed in pull-down assays (Supplementary Fig. S2). Taken toghether, our data indicate that AXL can bind to both p85 and GRB2. However, in thyroid cancer cells, it preferentially binds to p85.

### Human thyroid cancer specimens express Axl and Gas6

Twenty-seven thyroid carcinoma samples (9 PTCs, 10 FTCs, and 8 PDC/ATCs) were analyzed by immunohistochemical staining with an anti-human AXL antibody. Of all the samples

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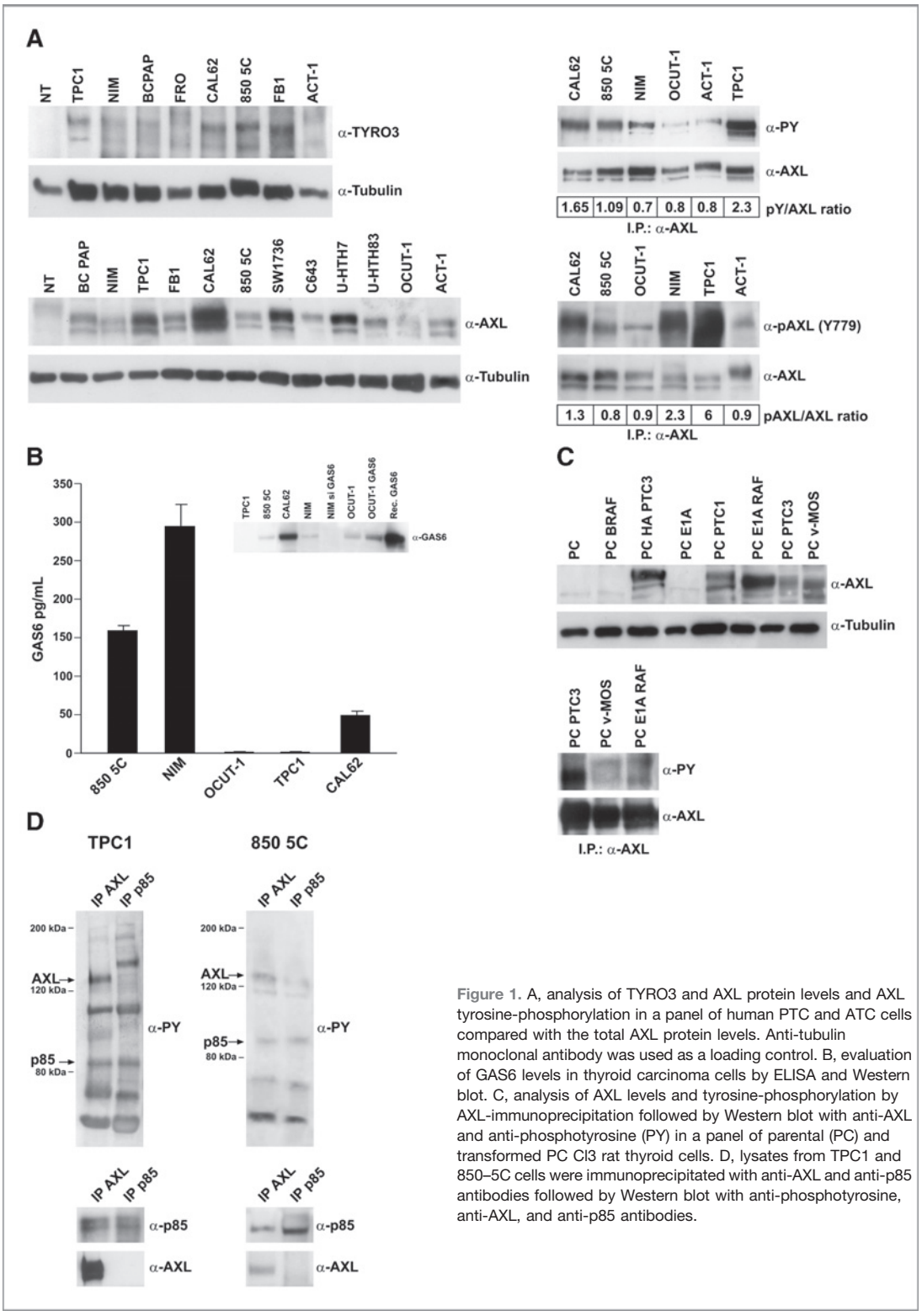


Figure 1. A, analysis of TYRO3 and AXL protein levels and AXL tyrosine-phosphorylation in a panel of human PTC and ATC cells compared with the total AXL protein levels. Anti-tubulin monoclonal antibody was used as a loading control. B, evaluation of GAS6 levels in thyroid carcinoma cells by ELISA and Western blot. C, analysis of AXL levels and tyrosine-phosphorylation by AXL-immunoprecipitation followed by Western blot with anti-AXL and anti-phosphotyrosine (PY) in a panel of parental (PC) and transformed PC Cl3 rat thyroid cells. D, lysates from TPC1 and 850–5C cells were immunoprecipitated with anti-AXL and anti-p85 antibodies followed by Western blot with anti-phosphotyrosine, anti-AXL, and anti-p85 antibodies.

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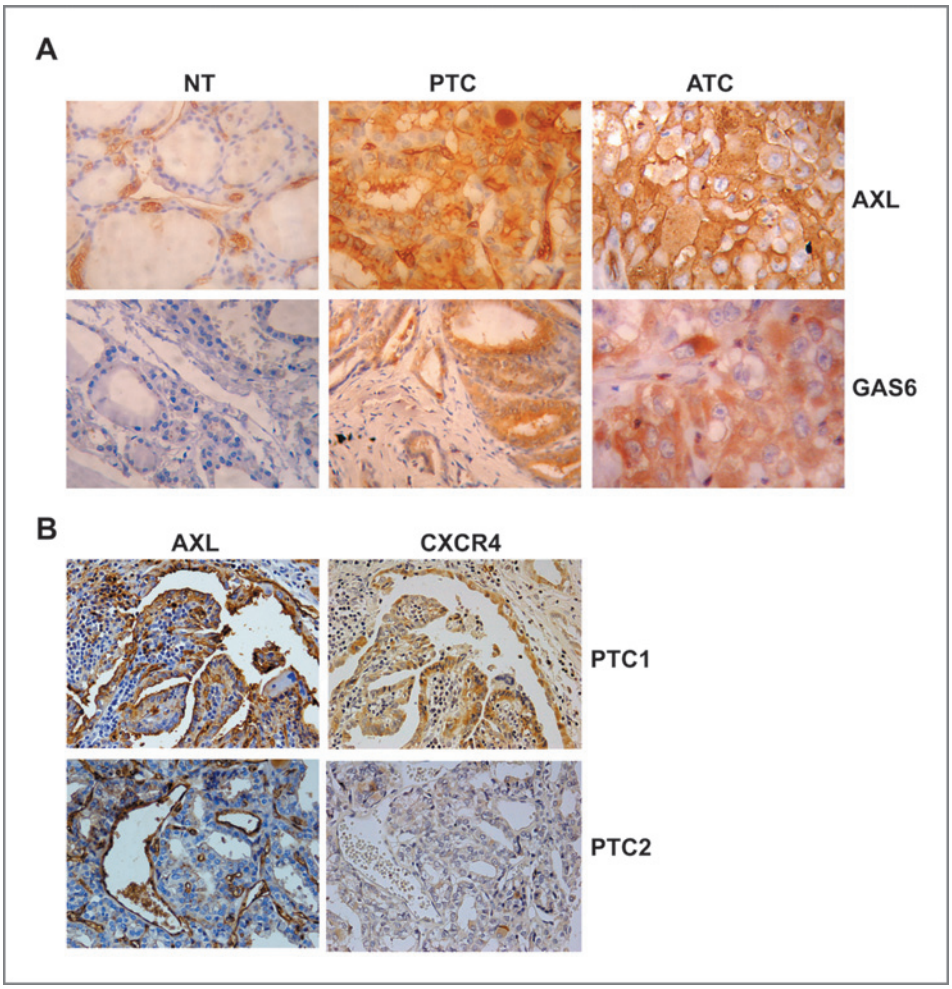


Figure 2. A, a representative example of immunohistochemistry on human thyroid carcinomas (PTC, ATC) and normal tissues (NT) with anti-AXL and anti-GAS6 antibodies. B, the same area of a human PTC immunostained for AXL and CXCR4 (PTC1). A negative sample is also shown (PTC2).

analyzed, 73% (19/27) scored positive for AXL expression. Normal thyroid was negative for AXL expression (Supplementary Table 1). AXL positivity was observed mainly in tumoral cells (Fig. 2A). Most of the samples displayed membrane positivity, but a small number of specimens showed cytosolic staining into perinuclear vescicles probably representing the Golgi apparatus, a typical pattern of overexpressed proteins. Tumoral stroma and nontumoral adjacent tissues were negative, with the exception of red blood cells that were strongly recognized by anti-AXL antibody as previously described (28). We also assessed GAS6 expression by IHC in the same sample set. Most of the analyzed specimens scored positive for GAS6 (Fig. 2A). GAS6 staining was cytosolic and mainly found in carcinoma cells; some samples also displayed stromal positivity, suggesting that the ligand can also be provided by the tumor microenvironment. As AXL was identified as a CXCR4-SDF-1 target, we analyzed an independent set of PTCs ($n = 30$) for both AXL and CXCR4 immunostaining (Fig. 2B). All the CXCR4-positive samples (10/30) also coexpressed AXL (10/10). AXL was expressed in 14 out of 30 samples. Thus, CXCR4 expression positively correlated with AXL immunostaining ($P < 0.005$).

### AXL/TYRO3-GAS6 blockade inhibits thyroid cancer cell proliferation and survival

We analyzed the level of DNA synthesis by BrdU incorporation assays in serum-deprived TPC1 cells with and without GAS6. Upon GAS6 stimulation, BrdU incorporation increased from 10% to 20%. Consistently, a chimaeric protein (Dtk/FC) that contains the extracellular domain of TYRO3 fused to the FC portion of the IgG that sequesters GAS6, did not modify the rate of BrdU-positive cells in basal conditions, but inhibited the effects of GAS6 treatment (Supplementary Fig. S4A). Then, we used RNA interference. AXL silencing was verified by Western blot (Fig. 3A) and FACS analysis (Supplementary Fig. S5). When AXL was silenced, we observed a reduction of BrdU incorporation both in basal conditions (Fig. 3A) and in the presence of GAS6: BrdU-positive cell rate dropped from 27% to 8%. GAS6 silencing did not modify TPC1 growth rate (not shown), consistent with the observation that GAS6 is not produced by these cells. To evaluate whether AXL blockade influenced TPC1 survival, we treated these cells with a proapoptotic substance, diethylmaleate, for 12 hours, and carried out TUNEL assays. In this condition, 48% of TUNEL-positive cells were observed; GAS6 addition dramatically decreased

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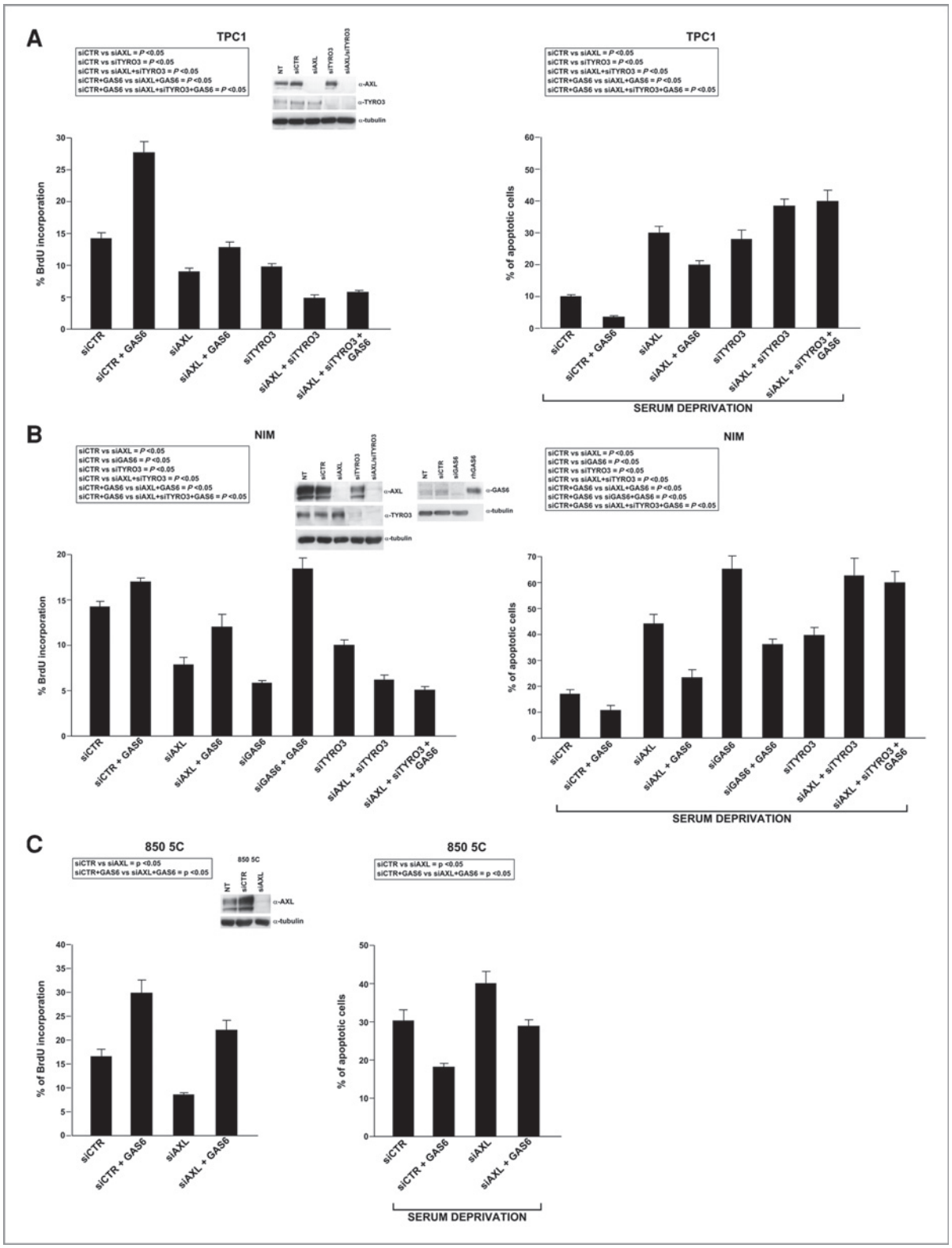


Figure 3. A, BrdU incorporation and TUNEL assay were evaluated in TPC1 cells after AXL and/or TYRO3 silencing in presence and in absence of GAS6. Western blot analysis to verify AXL and TYRO3 silencing is shown. Three independent experiments were carried out in which at least 400 cells were counted. B, BrdU incorporation and TUNEL assay of NIM cells treated or not with GAS6 and after AXL, GAS6, and TYRO3 silencing. Western blot analysis to verify AXL, GAS6, and TYRO3 silencing is shown. C, BrdU incorporation and TUNEL assay of 850–5C cells with and without GAS6 and after AXL silencing. Western blot analysis to verify AXL silencing is shown.

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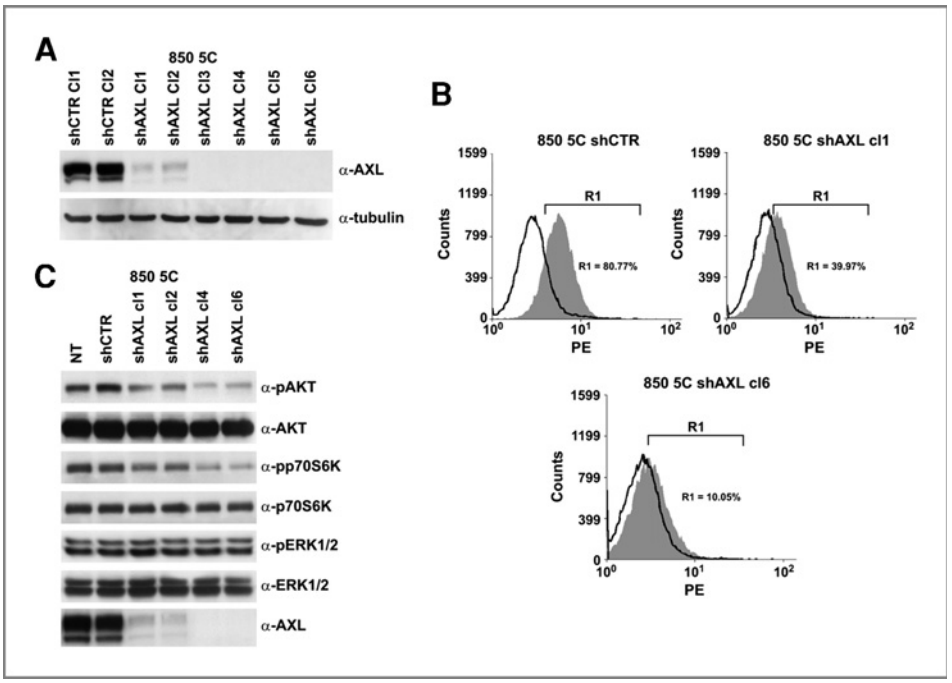


Figure 4. A, screening of 850–5C shAXL clones by Western blot with anti-AXL antibody. B, evaluation of AXL levels in selected shCTR and shAXL clones through FACS analysis. C, evaluation of AXL-mediated signaling pathways shCTR and shAXL clones with anti-phospho-AKT, -p70S6K, -ERK antibodies. Equal protein loading was verified with anti-AXL, -AKT, -p70S6K, -ERK antibodies. NT, normal tissue.

this percentage. Dtk/FC decreased the percentage of apoptotic cells only in the presence of exogenous GAS6 (Supplementary Fig. S4B). We also evaluated apoptosis in AXL-silenced cells. After 12 hours serum deprivation, 10% of apoptotic cells were observed. This percentage decreased to 5% when GAS6 was added. AXL silencing dramatically increased the rate of apoptotic cells to 30%. Interestingly, when GAS6 was added to AXL-silenced cells, the proapoptotic effects of AXL silencing were significantly inhibited (Fig. 3A). This effect could be due to incomplete AXL silencing. However, FACS analysis showed that RNAi almost completely suppressed AXL expression (Supplementary Fig. S5). Alternatively, TYRO3 can substitute AXL function. GAS6 silencing did not modify the TPC1 cell apoptotic rate (not shown). As the addition of GAS6 partially recovered the effects of AXL blockade in this cell line, we evaluated the effects of TYRO3 knockdown in TPC1 cells through RNA interference. As shown in Figure 3A, TYRO3 silencing affected both BrdU incorporation and apoptosis. Silencing of both receptors increased the effect of the single knockouts; finally, GAS6 treatment did not modify the effect of the double knockouts. Similar experiments were carried out by using NIM cells that express both AXL and GAS6. Exogenous addition of this ligand did not significantly modify BrdU incorporation and apoptosis rate of NIM cells. Instead, the blockade of both endogenous AXL and GAS6 by using RNAi or Dtk/FC inhibited NIM DNA synthesis, and exogenous GAS6 addition reverted these effects. NIM apoptosis induced by diethylmaleate or serum deprivation was only slightly modified by GAS6 treatment. However, Dtk/FC or AXL- or GAS6-silencing strongly enhanced NIM apoptotic rate, and these effects were again reverted by exogenous GAS6 (Fig. 3B and Supplementary Fig. S4). TYRO3 silencing also affected NIM proliferation and survival, and TYRO3/AXL silencing was as effective as GAS6; GAS6 treatment did not modify the effects of TYRO3/AXL silencing. GAS6, TYRO3, and AXL knockdown was verified by Western blot analysis (Fig. 3B). We also used siRNA to verify whether AXL knockdown could impair proliferation and survival of human 850–5C and Cal62 ATC cells. GAS6 stimulated cell proliferation and inhibited apoptosis both in 850–5C and in CAL62 cells and AXL silencing had the opposite effect (Fig. 3C, Supplementary Fig. S6).

### Effects of stable silencing of AXL in 8505-C ATC cells

We selected 850–5C cells for their ability to induce tumor formation in immunodeficient mice. We stably transfected these cells with a pool of vectors expressing 5 different shRNAs directed against AXL or expressing control nontargeting shRNAs. We identified clones that expressed intermediate (shAXL Cl 1 and 2) or low (shAXL Cl 3–6) AXL levels (Fig. 4A). These results were confirmed by FACS analysis (Fig. 4B). In 850–5C shAXL clones, the activation of the PI3-K pathway (phosphorylation of Akt and of p70S6 Kinase), was significantly reduced with respect to control or parental cells (Fig. 4C). In contrast, the phosphorylation of ERK1/2 was not affected by AXL silencing. We then verified whether AXL knockdown could impair cell growth both in optimal (10% FCS) or in low serum (2.5% FCS) conditions. AXL silencing showed mild effects on cell proliferation in complete culture medium (Supplementary Fig. S7), whereas it was significantly more effective at low serum concentration (Fig. 5A). To assess whether reduced cell growth was due to decreased DNA synthesis or increased cell death, cells were serum starved for 12 hours and the percentage of BrdU- and TUNEL-positive cells was evaluated. All shAXL clones displayed a significant reduction of BrdU incorporation and a significant increase in

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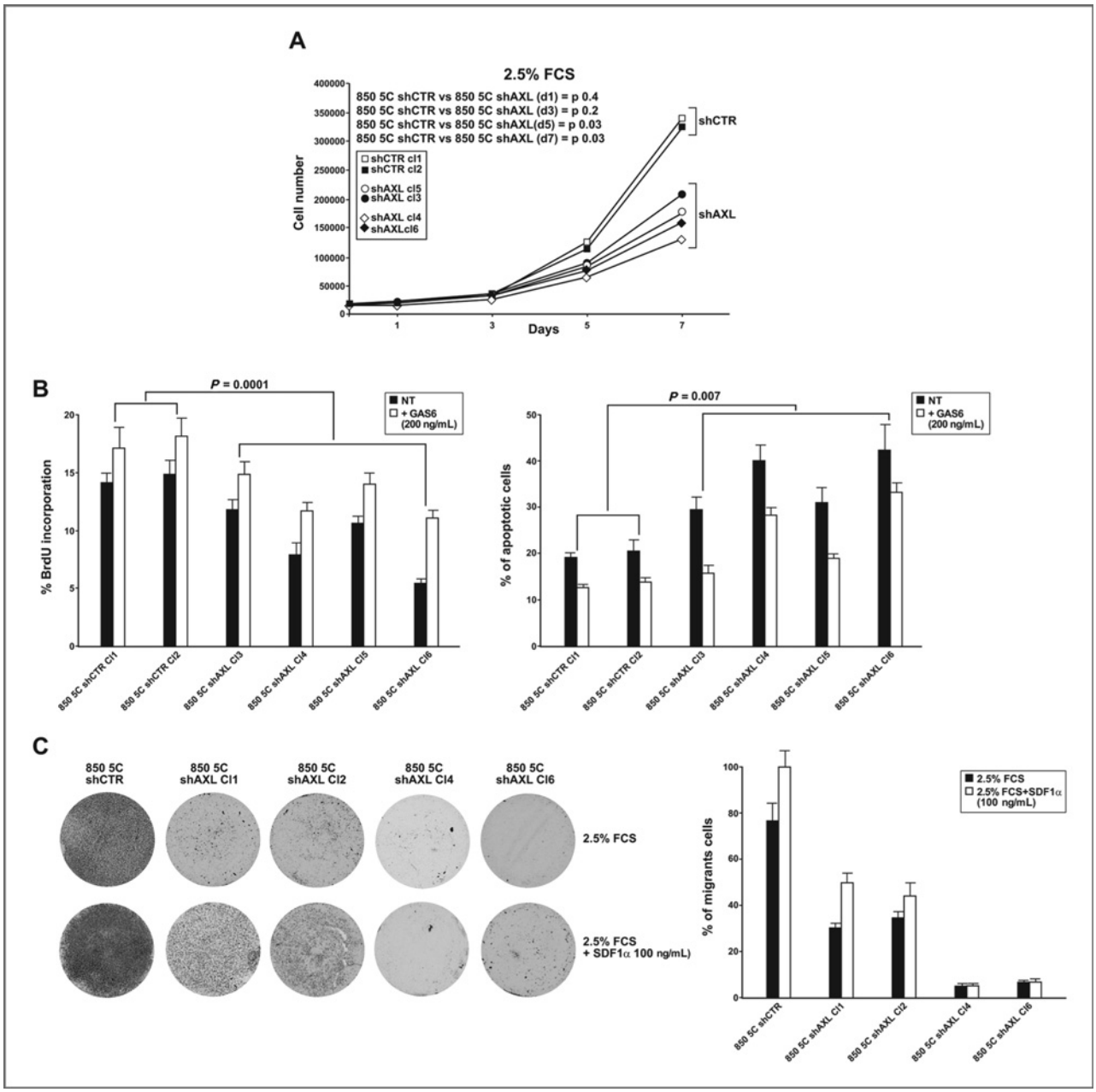


Figure 5. A, growth curves of 850–5C shCTR and shAXL clones done in 2.5% FBS. The average results of at least 3 independent determinations were reported. B, BrdU incorporation was measured to evaluate S-phase entry of 850–5C shAXL and shCTR clones both in the absence and in the presence of exogenous GAS6. TUNEL reaction was conducted on 850–5C shAXL and shCTR clones both in the presence and in the absence of exogenous GAS6. C, Matrigel invasion assay on 850–5C shAXL clones. The results were expressed as percentage of 850–5C shAXL migrated cells with respect to 850–5C shCTR.

apoptosis when compared with control clones, and these effects were partially reverted by exogenous GAS6 (Fig. 5B). Moreover, we asked whether AXL was necessary for ATC cell invasiveness through Matrigel both in the absence and in the presence of SDF-1α. shAXL clones showed a clear decrease of invasive ability with respect to shCTR cells (Fig. 5C). SDF-1α stimulated migration of cells displaying residual AXL expression, but was unable to do so in the AXL-negative clones, suggesting that AXL is required for SDF-1 α-mediated cell invasion of 850-5C cells. To rule out unspecific effects of the AXL shRNAs, we decided to inhibit AXL through an alternative strategy. We generated 850-5C cells expressing dominant-negative AXL proteins, a truncated form of human AXL, lacking the intracellular domain (AXL-EC), or a kinase-dead mutant, carrying the substitution K558R (AXL KD). Phosphotyrosine blotting confirmed inhibition of AXL signaling (Fig. 6A). AXL-EC and AXL KD inhibited AXL-mediated DNA synthesis, cell survival, and cell migration (Fig. 6B, C, and D).

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**Axl silencing inhibits experimental tumor growth**

Finally, we evaluated the role of AXL in tumor growth by using xenografts of ATC cells into (*nu/nu)* immunodeficient mice. We injected animals with parental, shCTR-transfected (shCTR), and shAXL-expressing (Cl4 and Cl6) 850–5C cells, and compared their growth rate. Parental cells formed tumors with the same efficiency as shCTR-transfected cells did (not shown). As shown in Fig. 7A, the tumorigenicity of 850–5C shAXL clones was completely inhibited when compared with 850–5C shCTR control cells. At 3 weeks, shCTR tumor median volume was 34 $mm^3$, whereas shAXL was lesser than 10 $mm^3$. At 4, 5, and 6 weeks, shCTR tumors continued to grow, reaching a median volume of approximately 150 $mm^3$ whereas shAXL did not, and the few small tumors that appeared at 3 weeks eventually regressed. At the end of the experiment, no shAXL tumors were available. This precluded histological analysis. We then tested the ability of 850–5C shAXL clones with intermediate levels of AXL expression to induce tumor formation in nude mice. We injected mice with shAXL Cl2, that still retains some expression of the receptor (Fig. 7B), and, as control, the shCTR clone. A few small tumors appeared at 7 weeks postinjection in the shAXL Cl2 group (Fig. 7B). At the end of the experiment, tumors were excised and analyzed by IHC for proliferation, apoptosis, and vascularization. A higher rate of apoptosis, but not of proliferation of shAXL tumors with respect to controls was observed, although differences were not statistically significant due to the limited number of shAXL samples (Fig. 7C). Vessel density was comparable between the 2 groups of tumors. However, in shAXL tumors, vessels were not well formed and their median diameter was smaller than that of shCTR clones ($P < 0.05$). These data indicate that AXL is required for 850–5C xenograft growth in nude mice.

## Discussion

In the search for SDF-1 transcriptional targets in thyroid cancer, we identified 2 tyrosine-kinase receptors, TYRO3 and AXL. AXL overexpression has been reported in a variety of human cancers (21, 24, 28–36), and it is associated with negative prognosis (14, 24, 25, 37–39). AXL expression confers resistance to different antineoplastic agents (40–42), can induce angiogenesis (14, 43), cancer cell migration and invasion (37, 44–46).

We found that *TYRO3* and *AXL* are transcriptionally regulated by CXCR4-SDF-1. However, these receptors were constitutively expressed in human thyroid cancer cells, but not in normal thyroid. AXL expression was also detected in thyroid cancer samples, but not in normal thyroid tissues, and the presence of AXL positively correlated with CXCR4 expression. Rat thyroid cancer cells (PC) expressing different activated oncogenes also expressed AXL, with the exception of PC BRAF and PC E1A. These data indicate that multiple mechanisms, besides CXCR4 activation, can contribute to increase AXL levels in thyroid cancer. The activation of components of the RAS/MAPK pathway is frequently found in human PTC. However PC PTC, but not PC BRAF cells, express AXL, despite both display activated MAPKs. This suggests that the RAS/MAPK pathway is not sufficient to activate AXL expression. Cancer-associated transcription factors, promoter hypomethylation or gene amplification may result in *AXL* overexpression. Interestingly, AXL is strongly induced by epithelial-to-mesenchimal transition (37) or by a v-FLIP-NFKB pathway (47). Preliminary data obtained by us failed to show gene mutation or amplification in thyroid cancer cell lines (Krishnamoorthy, unpublished observations). However, other possible mechanisms of AXL expression in thyroid cancer need to be investigated. AXL was constitutively phosphorylated in most of the human thyroid cancer cells. This was due to the presence of the ligand GAS6; the only exception was the TPC1 cell line, in which AXL was highly phosphorylated despite ligand absence. In this case, some other mechanisms, such as Ret-mediated ligand-independent activation of AXL, may keep the receptor in a constitutively active status. Consistently, a cross-talk between EGFR family members and AXL has been suggested (38, 42). The inhibition of TYRO3, AXL, or GAS6 reduced cell proliferation and increased apoptotic rate. AXL silencing in 850–5C ATC cells dramatically reduced thyroid cancer cell invasive ability, and SDF-1 could not rescue this capability, indicating that AXL is required for SDF-1-mediated chemotactic activity. These results are in accord with previous data indicating that AXL is involved in cell migration and invasion (37, 44). AXL is also involved in angiogenesis (14, 43). Accordingly, in our xenograft system, AXL silencing strongly inhibited 850–5C tumor formation, and this effect was mainly due to impaired vessel formation in xenografts. We also observed increased apoptosis in 850–5C shAXL induced tumors with respect to the normal counterpart. However, differences in the apoptotic rate were not statistically significant. Thus, in our thyroid cancer model, AXL seems to be required for the initial steps of tumor formation. Other studies have shown that AXL is required for breast cancer cell xenograft growth in nude mice, but it was not required for orthotopic breast tumor formation. However, it was necessary for invasion and metastasis (37). Differences in AXL requirement between subcutaneously- or orthotopically-injected breast tumors suggested a specific function for this receptor to drive growth and colonization in an unfavorable environment (48, 49). Whether orthotopic thyroid tumor formation in syngeneic animals requires AXL is currently unknown. In breast cancer, AXL expression has been associated with the presence of metastasis and it represents a negative prognostic factor (37). We have analyzed a limited number ($n = 27$) of thyroid cancer samples, ranging from the well-differentiated PTC and FTC to the completely undifferentiated ATC, and we found that AXL expression was equally distributed in all 3 histotypes. This analysis, although done on a small number of patients, indicates that AXL expression may be an initial event of thyroid transformation, but it is sometimes retained during thyroid cancer progression. TYRO3, often coexpressed with AXL in thyroid cancer cells, is also important for thyroid cancer cell viability. However, tumorigenesis in nude mice is strictly dependent on AXL.

In conclusion, our data strongly suggest that AXL/TYRO3-GAS6 axis can be considered as a novel potential target of thyroid anticancer therapy. Several compounds have been identified that block AXL signaling by acting at different levels

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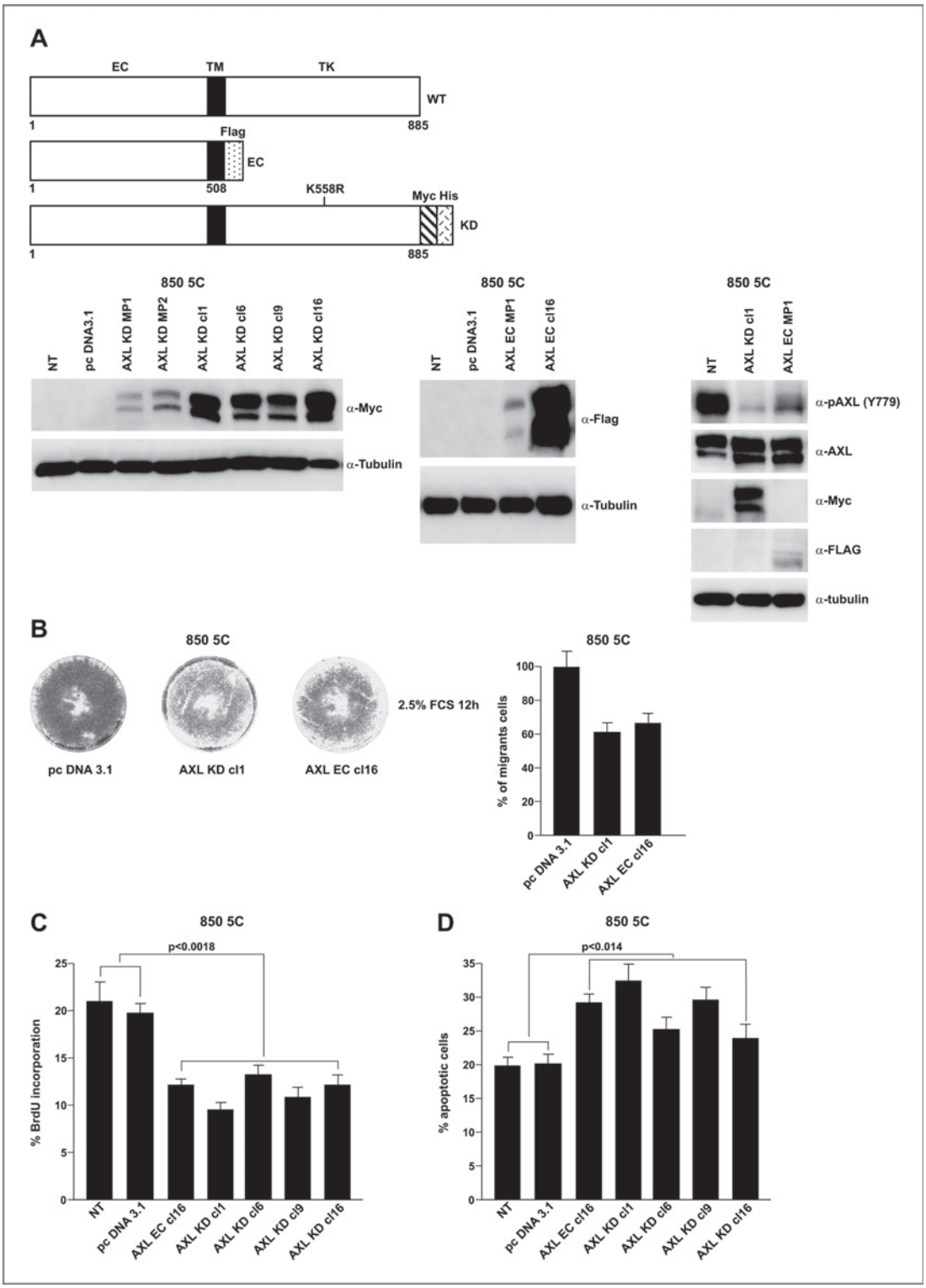


Figure 6. A, schematic representation of wt and mutant AXL. AXL-EC includes Aa 1 to 508, whereas AXL KD contains the substitution K558R. FLAG and myc-his epitopes are indicated. Exogenous AXL KD and AXL EC expression in different clones was verified by Western blot analysis with anti-myc and anti-FLAG antibodies, respectively. 850–5C AXL KD cl 1 and AXL EC MP1 were also analyzed for AXL phosphorylation through anti-phospho-AXL immunoblot. B, Matrigel invasion assay on control (pcDNA3.1), AXL KD- and AXL EC- 850–5C cells. The results were expressed as the percentage of migrating cells with respect to controls. C, BrdU incorporation and TUNEL assays were carried out to evaluate S-phase entry and apoptosis of 850–5C (NT), empty vector-transfected (pcDNA3.1), and AXL mutant clones. The percentage of apoptotic cells was measured by TUNEL assay in 850–5C (NT), empty vector-transfected (pcDNA3.1), and AXL mutant clones. NT, normal tissue.

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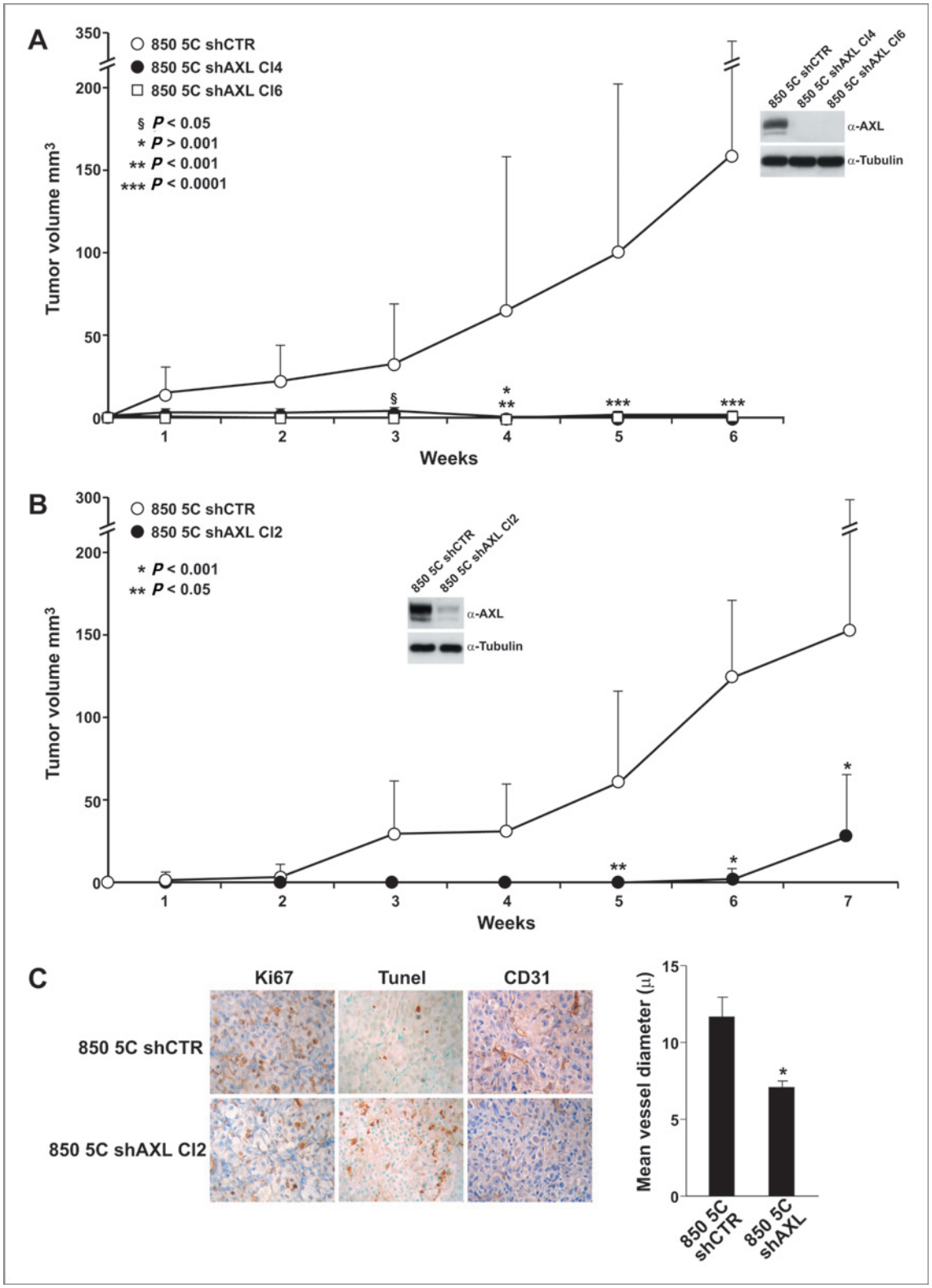


Figure 7. A, three groups of 10 nude mice injected with 850–5C shCTR or 850–5C shAXL CL4 or CL6 cells were evaluated for tumor growth. All *P* values were 2-sided, and differences were statistically significant at $P < 0.05$. B, 2 groups of 10 nude mice injected with 850–5C shCTR cells or 850–5C shAXL Cl2 that expressed intermediate levels of AXL were evaluated for tumor growth. Mice inoculated with shAXL Cl2 formed tumors after 7 weeks, but their size was smaller than that of shCTR tumors. C, IHC on excised shAXL Cl2 and shCTR tumors for ki67, CD31, and Tunel. Mean vessel diameter was significantly lower in shAXL than in control clones; $^*P < 0.02$.

(43, 44, 49, 50). Our data indicate that these compounds should be exploited to treat thyroid cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Ackonwledgments

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