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TYRO3 as a molecular target for growth inhibition and apoptosis induction in bladder cancer

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Running title: TYRO3 as a therapeutic target in bladder cancer

ABSTRACT

BACKGROUND: Muscle-invasive bladder cancer (MIBC) is an aggressive neoplasm with poor prognosis, lacking effective therapeutic targets. Oncogenic dependency on members of the TAM tyrosine kinase receptor family (TYRO3, AXL, MERTK) has been reported in several cancer types, but their role in bladder cancer has never been explored.

METHODS: TAM receptor expression was evaluated in two series of human bladder tumors by gene expression (TCGA and CIT series), immunohistochemistry or western blotting analyses (CIT series). The role of the different TAM receptors was assessed by loss-of-function experiments and pharmaceutical inhibition *in vitro* and *in vivo*.

RESULTS: We reported a significantly higher expression of TYRO3, but not AXL or MERTK, in both non-MIBCs and MIBCs, compared to normal urothelium. Loss-of-function experiments identified a TYRO3-dependency of bladder carcinoma-derived cells both *in vitro* and in a mouse xenograft model, whereas AXL and MERTK depletion had only a minor impact on cell viability. Accordingly, TYRO3-dependent bladder tumor cells were sensitive to pharmacological treatment with two pan-TAM inhibitors. Finally, growth inhibition upon TYRO3 depletion relies on cell cycle inhibition and apoptosis associated with induction of tumor-suppressive signals.

CONCLUSIONS: Our results provide a preclinical proof of concept for TYRO3 as a potential therapeutic target in bladder cancer.

1 BACKGROUND

2 Bladder cancer is the ninth most common cancer worldwide, with approximately 430,000
3 new cases diagnosed in 2012 and 165,000 deaths annually ¹. Muscle-invasive bladder
4 cancers (MIBCs) account for 25% of bladder tumors at initial diagnosis and are life-
5 threatening. Indeed, despite radical cystectomy with cisplatin-based neoadjuvant and/or
6 adjuvant chemotherapy, the standard treatment for MIBC, overall survival is only about
7 50% at five years, and is as low as 5% in cases of distant metastasis. Immune
8 checkpoint inhibitors can be beneficial in patients with bladder cancer ², but they are
9 effective in only a limited number of patients (20%). New effective therapies targeting
10 both MIBCs and non-muscle-invasive tumors (NMIBCs), which often recur, are therefore
11 required. Efforts to decipher the molecular mechanisms involved in bladder
12 carcinogenesis have led to the identification of a number of possible new treatment
13 targets, such as mTOR in patients with *TSC1* mutations, epidermal growth factor
14 receptor 2 (HER2)/ERBB2 in HER2-positive tumors, EGFR in basal-like tumors, and
15 fibroblast growth factor receptors, particularly in patients harboring mutations or gene
16 fusions of *FGFR3* ³⁻⁵.

17
18 The TAM family of receptor tyrosine kinases (RTKs), which includes TYRO3, AXL and
19 MERTK, has emerged as new therapeutic targets in many types of cancer, but their role
20 in bladder cancers has not yet been determined. TAM receptors can be activated by the
21 vitamin K-dependent protein GAS6 (growth arrest-specific 6), but ligand-independent
22 activation has also been described ⁶. TAM receptors are involved in immune control,
23 inflammation and homeostasis in several organs, including the nervous, reproductive
24 and vascular systems ^{7,8}. Aberrant TAM receptor expression has been reported in

diverse solid and hematopoietic tumors in humans, with effects on apoptosis, growth, metastasis and chemosensitivity, although the underlying mechanisms remain incompletely understood⁸⁻¹⁰. AXL has been, so far, more extensively studied in cancers than either MERTK or TYRO3.

In this study, we investigated TAM receptors expression in two independent cohorts of bladder tumors and evaluated the influence of each receptor on the regulation of growth/survival in urothelial bladder cancer-derived cell lines (UBC lines).

MATERIALS AND METHODS

Cell lines

The human bladder cancer-derived cell lines RT112, UM-UC-5, UM-UC-9, VM-CUB-1, 5637, 647V and HT1376 were obtained from DSMZ (Heidelberg, Germany). MGH-U3 cells were kindly provided by Dr. Francisco X. Real (CNIO, Madrid). MGH-U3, UM-UC-5, UM-UC-9, VM-CUB-1, 647V and HT1376 cells were cultured in DMEM, whereas RT112, and 5637, cells were cultured in RPMI. Media were supplemented with 10% fetal calf serum (FCS) (ThermoFisher Scientific, Courtaboeuf, France). Cells were kept at 37°C, under an atmosphere containing 5% CO₂. The identity of the cell lines used was checked by analyzing genomic alterations on comparative genomic hybridization arrays (CGH array) and sequencing genes known to be mutated: *RAS*, *TP53*, *FGFR3* and *PIK3CA*. The cells were routinely checked for mycoplasma contamination.

Human bladder samples

We assessed expression of the *TYRO3*, *AXL*, *MERTK* and *GAS6* genes by RT-qPCR, using 169 bladder tumor samples (87 NMIBCs and 82 MIBCs) from the previously described CIT-series cohort ("Carte d'Identité des Tumeurs" or "Tumor identity card") of bladder tumors^{5,11}. Seven normal urothelial samples were obtained from fresh urothelial cells scraped from the normal bladder wall and dissected from the lamina propria during organ procurement from a cadaveric donor for transplantation. RNA, DNA and protein were extracted from the surgical samples by cesium chloride density centrifugation, as previously described^{5,12}. We used protein extracted from 21 human bladder tumors from the CIT-series (4 NMIBCs and 17 MIBCs) for western-blot analysis^{5,12}. Lyophilized proteins were solubilized in 1X Laemmli sample buffer and boiled for 10 minutes. Protein concentrations were determined with the BioRad Bradford Protein Assay Kit (BioRad, Marnes-la-Coquette, France) and TAM protein levels were assessed by immunoblotting.

RNA extraction and real-time reverse transcription-quantitative PCR

RNA was isolated from cell lines and xenografts with RNeasy Mini kits (Qiagen, Courtaboeuf, France). Reverse transcription was performed with 1 µg of total RNA, and a high-capacity cDNA reverse transcription kit (ThermoFisher Scientific). A predesigned assay was used to quantify expression of the TATA-box binding protein (*TBP*) reference gene (ThermoFisher Scientific, Ref: 4326322E). Custom-designed assays were used to measure expression of the *TYRO3*, *AXL*, *MERTK* and *GAS6* genes. Primers and probes were designed with Probe Finder software at the Universal Probe Library Assay Design Center (Roche). RT-qPCR settings were as described elsewhere⁵. For each gene of interest, the amount of mRNA was normalized against that for the *TBP* reference gene by the $2^{-\Delta\Delta C_t}$ method.

1 **TYRO3 (Roche Universal Probe Library probe ID: 14):**

2 5'- GAGGATGGGGGTGAAACC-3' (sense strand)

3 5'- ACTGTGAAAAATGGCACACCT-3' (antisense strand)

4 **AXL (Roche Universal Probe Library probe ID: 76):**

5 5'- AACCAGGACGACTCCATCC-3' (sense strand)

6 5'- AGCTCTGACCTCGTGCAGAT-3' (antisense strand)

7 **MERTK (Roche Universal Probe Library probe ID: 6):**

8 5'- ATTGGAGACAGGACCAAAGC-3' (sense strand)

9 5'- GGGCAATATCCACCATGAAC-3' (antisense strand)

10 **GAS6 (Roche Universal Probe Library probe ID: 17):**

11 5'-ATGGCATGTGGCAGACAAT-3' (sense strand)

12 5'-CCCTGTTGACCTTGATGACC-3' (antisense strand)

13

14 **Immunohistochemistry**

15 Formalin-fixed, paraffin-embedded 3 µm tissue sections of tumors from the CIT-series
16 were placed on poly-L-lysine coated slides. The paraffin was removed by immersion in
17 xylene and the section was rehydrated by immersion in a graded series of alcohol
18 concentrations. Antigens were retrieved by heating sections at 95°C in 10 mM citrate
19 buffer pH 9 (Microm Microtech France, Brignais, France) for 20 min. Endogenous
20 peroxidase activity was inhibited by incubation in 3% H₂O₂. The section was then
21 incubated in Quanto Protein Block solution (Microm Microtech France) for 1 h to
22 minimize nonspecific staining. The sections were then incubated with a rabbit polyclonal
23 anti-TYRO3 antibody (Ref: HPA071245, Sigma-Aldrich, Saint-Quentin Fallavier, France)
24 diluted 1:50 in antibody diluent solution (Diamond antibody diluent, Cell Marque, Rocklin,

USA) for 1 h at 37°C. Antibody binding was detected with N-Histofin® Simple staining and a DAB detection kit (Microm Microtech France), according to the manufacturer's instructions. Antibody specificity for TYRO3 was assessed with formalin-fixed paraffin-embedded 5637 bladder cancer cells, which express TYRO3, AXL and MERTK, after transfection with either control siRNA or *TYRO3* siRNA#2.

Flow cytometry

Bladder cancer cells (1×10^6 cells/condition) were collected with Accutase (Sigma-Aldrich), washed in cold phosphate-buffered saline (PBS), and incubated with 1 µg of anti-TYRO3 clone 5B4 (Ref: GTX83459, GeneTex, Irvine, USA) or isotype control (Ref: MAB003 R&D Systems, Lille, France) antibody in PBS supplemented with 0.5% bovine serum albumin (BSA) for 45 minutes on ice. The cells were then washed three times in cold 0.5% BSA PBS and incubated for 30 minutes on ice with 2 µg/ml phycoerythrin-conjugated goat-anti-mouse Ig (Ref: 550589, BD Biosciences, Le Pont de Claix, France) in 0.5% BSA PBS. Cells were washed three times in 0.5% BSA PBS and the fluorescence signal was acquired with a BD LSR II flow cytometer (BD Biosciences).

Cell viability after transfection with siRNA and treatment with small compounds

For siRNA transfection, cells were transfected with either 20 nM (MGH-U3, RT112, UM-UC-5, UM-UC-9, 5637 and 647V) or 5 nM (VM-CUB-1) siRNA in the presence of Lipofectamine RNAi Max reagent (ThermoFisher Scientific), in accordance with the manufacturer's protocol. siRNAs were purchased from Ambion and Qiagen. For the control siRNA, we used a Qiagen control targeting luciferase (Ref: SI03650353). The sequences of the siRNAs are described in Table S1.

For small-compound inhibitor treatments, MGH-U3, RT112, UM-UC-5, UM-UC-9 and VM-CUB-1 cells were used to seed 96-well plates with appropriate culture media supplemented with 10% FCS. After 24 h, the cells were treated with either UNC-2025 (Ref. S7576, Euromedex, Souffelweyersheim, France) or BMS-777607 (Ref. HY-12076, CliniSciences, Nanterre, France).

Cell viability was assessed with the CellTiter-Glo assay (Promega, Charbonnières-les-Bains, France).

Protein extraction and immunoblotting

Cell lysates were prepared and immunoblotting were carried out as previously described¹³. Anti-TYRO3 (Ref. 5585), AXL (Ref. 8661), MERTK (Ref. 4319), caspase-8 (Ref. 9746), cleaved caspase-3 (Ref. 9664), cleaved PARP (Ref. 5625), FOXM1 (Ref. 5436), pRB (Ref. 9309), phospho-pRB Ser780 (Ref. 9307), CYCLIN D1 (Ref. 2926), AURORA A (Ref. 14475), AURORA B (Ref. 3094), SURVIVIN (Ref. 2808), c-MYC (Ref. 9402), anti-mouse IgG, HRP-linked (Ref. 7076) and anti-rabbit IgG, HRP-linked (Ref. 7074) antibodies were purchased from Cell Signaling Technology (Ozyme, Montigny-le-Bretonneux, France). Anti- α -TUBULIN (Ref. T6199) and anti- β -ACTIN (Ref. A2228) antibodies were obtained from Sigma-Aldrich. For peptide-N-glycosidase F (PNGaseF), we digested 10 μ g of protein with PNGaseF, according to the manufacturer's instructions (New England Biolabs, Evry, France).

Soft agar assay

MGH-U3 and RT112 cells, untransfected or transfected with siRNA, were used to seed 12-well plates containing DMEM supplemented with 10% FCS and 1% agar, in triplicate

(20,000 cells/well). The medium was changed weekly. The plates were incubated for 14 days and colonies larger than 50 μm in diameter, as measured with a phase-contrast microscope equipped with a measuring grid, were counted.

DNA array

For the identification of genes displaying changes in expression after *TYRO3* knockdown in MGH-U3, RT112 and UM-UC-5 cells, we transfected the cells for 40 h with *TYRO3* siRNA#1, *TYRO3* siRNA#2 or *TYRO3* siRNA#3. mRNA was extracted and purified with RNeasy Mini kits (Qiagen). Total RNA (200 ng) from control and siRNA-treated MGH-U3, RT112 and UM-UC-5 cells was analyzed with the Affymetrix U133 Plus 2.0 DNA array. Raw gene expression data were normalized and summarized by the RMA (robust multi-array averaging) method (R package affy) with a customized chip definition developed by Microarray Lab, BrainArray (HGU133Plus2_Hs_ENTREZG_v18)^{14,15}. One log₂-transformed signal value per gene was obtained. The microarray data described here are available from GEO (<https://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE100025. The LIMMA algorithm was used to identify genes differentially expressed between *TYRO3* siRNA-treated ($n=9$) and Lipofectamine-treated control ($n=9$) cells¹⁶. The p -values were adjusted for multiple testing by the Benjamini–Hochberg FDR procedure. Genes with $|\log_2\text{FC}| \geq 0.58$, and with a FDR below 5% were considered to be differentially expressed.

Xenograft models

1 Six-week-old female Swiss *nu/nu* mice (Charles River Laboratories, Saint-Germain-
2 Nuelles, France) were raised in the animal facilities of Institut Curie, in specific
3 pathogen-free conditions. They were housed and cared for in accordance with the
4 institutional guidelines of the French National Ethics Committee (*Ministère de*
5 *l'Agriculture et de la Forêt, Direction de la Santé et de la Protection Animale*, Paris,
6 France), under the supervision of authorized investigators. Mice received a
7 subcutaneous injection, into each flank (dorsal region), of 5×10^6 MGH-U3 bladder cancer
8 cells in 100 μ l PBS. For each study, with each of the cell lines, mice were randomly
9 separated into groups of six mice when tumors reached a volume of $50 \text{ mm}^3 (\pm 5)$. The
10 mice bearing MGH-U3 xenografts were treated three times weekly, for three weeks, with
11 an intraperitoneal injection of 4 μ g siRNA (*TYRO3* siRNA#1 or corresponding Scramble
12 siRNA) or of vehicle (PBS). Tumor size was measured twice weekly with a caliper, and
13 tumor volume in mm^3 was calculated as follows: $\pi/6 \times (\text{largest diameter}) \times (\text{shortest}$
14 $\text{diameter})^2$. The tumors were removed at the end of treatment. Part of the tumor was
15 flash-frozen in liquid nitrogen for mRNA extraction. The rest was fixed in 4% formol and
16 embedded in paraffin.

18 **TUNEL assay**

19 DNA fragmentation was evaluated with a TUNEL (deoxynucleotidyl transferase (Tdt)-
20 mediated nick-end labeling) assay detection kit (Sigma-Aldrich), according to the
21 manufacturer's instructions.

23 **Statistical analysis**

1 TAM expression in tumors was analyzed with Mann-Whitney tests. Linear Models for
2 Microarray Data (LIMMA) ¹⁶ was used to analyze DNA array experiments involving
3 simultaneous comparisons between large numbers of RNA targets. All functional
4 experiments were carried out twice or three times, in triplicate. For the comparison of
5 data between cell lines, two-tailed *t*-tests were used. The control siRNA (Luciferase GL2
6 siRNA) or DMSO group was used as the reference group. Data are expressed as means
7 $\pm$ SD, and differences with a *p* value < 0.05 were considered statistically significant. For
8 the comparison of data between xenograft treatments, the non-parametric Mann-
9 Whitney test was used, with the vehicle group as the reference group. All statistical
10 analyses were performed with Prism7.0b (GraphPad Software, Inc).

11

12 **RESULTS**

13 **TYRO3 is highly expressed in bladder carcinomas**

14 We first investigated the role of TAM receptors in human bladder carcinoma, by
15 analyzing, using RT-qPCR, the levels of expression of *AXL*, *MERTK*, *TYRO3* and *GAS6*
16 in our previously described CIT cohort of 169 bladder tumors encompassing 87 NMIBCs
17 and 82 MIBCs ^{5,11}. Seven normal urothelium samples were used as controls (Figure 1A).
18 Expression levels for the three receptors and their ligand were heterogeneous in tumors,
19 with only *TYRO3* significantly elevated in NMIBCs and MIBCs relative to normal
20 samples. *GAS6* was more strongly expressed in MIBCs than in NMIBCs, suggesting that
21 *GAS6* might increase the autocrine or paracrine activation of *TYRO3* in muscle-invasive
22 tumors (Figure 1A). Higher *TYRO3* expression in MIBCs relative to normal samples was
23 confirmed by an analysis of publicly available RNA-seq data from the TCGA cohort for
24 405 MIBCs (primary tumors only) and 14 normal samples ¹⁷ (Supplementary Figure S1).

1 As in the CIT cohort, no increased expression of *AXL*, *MERTK* or *GAS6* was observed in
2 MIBCs compared to normal samples but surprisingly in this data set a decreased of
3 *GAS6* and *AXL* mRNA levels could even be observed. This discrepancy could be due to
4 the difference of so-called normal samples in the two series: pure normal urothelium
5 samples in the CIT cohort, versus peritumoral bladder tissues with a normal histological
6 appearance in the TCGA cohort. The difference in the number of samples considered
7 could also influence the results. We further analyzed TAM receptor expression at the
8 protein level, by western blots on a subset of tumors from our CIT cohort (Figure 1B and
9 Supplementary Figure S2). Consistent with the results of mRNA analyses, TYRO3 levels
10 in almost all tumors were higher than those in normal urothelium and vesical muscle
11 samples, whereas AXL levels were higher than those in the urothelium in only a few
12 tumors and this protein was abundant in the vesical muscle. However, strong MERTK
13 expression was surprisingly detected in 8 of 21 tumors. Different migration profiles were
14 observed for TYRO3, possibly due to post-translational modifications of the receptor
15 (Figure 1B). An analysis of the TYRO3 sequence with NetNGlyc1.0 software identified
16 seven potent N-glycosylation sites (not shown). The treatment of two tumor protein
17 samples with peptide N-glycanase F (PNGaseF), an enzyme that removes N-linked
18 glycans, shifted the TYRO3 band to the expected theoretical molecular weight of 96
19 kDa, confirming the N-glycosylation of this receptor (Figure 1C). We examined the
20 protein expression pattern of TYRO3 in clinical samples from the CIT cohort by
21 immunohistochemistry experiments using an anti-TYRO3 antibody. We had validated
22 the specificity of the antibodies using paraffin embedded 5637 cells expressing the three
23 TAM receptors and that were transfected or not with *TYRO3* siRNA (Supplementary
24 Figure S3). No or weak TYRO3 staining was observed on urothelium from histologically

normal tissue adjacent to the tumor (NAT), whereas TYRO3 was highly expressed by tumor epithelial cells and not by stromal cells in both NMIBCs and MIBCs (Figure 1D). Strikingly but as previously observed in colorectal tumor cells ¹⁸, TYRO3 staining was mainly detected in the cytoplasm of tumor cells and also at the plasma membrane. Overall, these results suggest that TYRO3 may play a role in bladder tumors.

TAM receptors are expressed in bladder cancer cell lines, but TYRO3 is the main TAM involved in modulating cell viability

We then investigated the protein expression of the TAM receptors, by western-blot, in a panel of cell lines derived from human urothelial bladder cancer (UBC) (Figure 2A). Consistent with our results for human bladder tumors (Figure 1B), TYRO3 was the TAM receptor most frequently expressed in UBC cell lines (21 of 25 cell lines), followed by AXL (13/25) and MERTK (7/25). Most of the UBC cell lines strongly expressed at least two TAM receptors, but MGH-U3 and BC3C expressed only TYRO3 (Figure 2A). We then used a loss-of-function approach based on siRNA to investigate whether TAM receptors were required for cell growth/survival (Figure 2B). We investigated the potential redundancy and relative importance of the three receptors, by selecting one cell line expressing all three TAM receptors (VM-CUB-1), three cell lines expressing TYRO3 together with AXL or MERTK (RT112, UM-UC-5 and UM-UC-9) and one cell line expressing only TYRO3 (MGH-U3) (Figures 2A and 2B). Western-blot analysis showed that each targeted TAM was effectively silenced 72 hours after transfection and that the knock-down of one TAM receptor had mostly no effect on the other TAM receptors with the exception of *TYRO3* knock-down using siRNA#1 that reduced AXL and increased MERTK expression, particularly in RT112 (Supplementary Figure S4). *TYRO3*

1 knockdown significantly and strongly decreased cell viability in all the cell lines tested,
2 regardless of the expression levels of other TAM receptors (60% to 75%, 96 hours after
3 transfection, depending on the cell line), demonstrating the high dependence for growth
4 of bladder cancer cell lines on TYRO3 expression and the absence of redundancy with
5 the other TAM receptors (Figure 2B and Supplementary Table S2). Moreover, when
6 TYRO3 was co-expressed with other TAM receptors, the effects of *TYRO3* silencing
7 were consistently more important than those of *AXL* or *MERTK* knockdown, highlighting
8 the predominant role of TYRO3 in the modulation of bladder cancer cell viability and
9 suggesting the potential for therapeutic targeting of TYRO3 (Figure 2B). This inhibition of
10 TYRO3 could be potentially achieved specifically using blocking antibodies as flow
11 cytometry experiments highlighted TYRO3 expression at the cell surface of the TYRO3-
12 dependent cell lines (MGH-U3, UM-UC-5 and RT112 (Supplementary Figure S5)).
13 However, as no such blocking antibodies were commercially available, we carried out
14 TYRO3 inhibition experiments using two pan TAM receptor inhibitors, UNC-2025 (IC₅₀
15 values are 18 nM, 7.5 nM and 0.7 nM for TYRO3, AXL and MERTK, respectively) and
16 BMS-777607 (IC₅₀ values are 4.3 nM, 1.1 nM and 14 nM for TYRO3, AXL and MERTK,
17 respectively)^{9,19,20}. These molecules are also described to inhibit with high affinity two
18 other tyrosine kinase receptors, MET (IC₅₀: 3.9 nM and 364 nM) and FLT3 (IC₅₀: 16 nM
19 and 0.35 nM); IC₅₀ given for BMS-777607 and UNC-2025 respectively^{19,20}. Consistent
20 with their affinity profile for the TAM receptors, treatment of VM-CUB-1 cells with 5 µM of
21 BMS-777607 or UNC-2025 inhibit the phosphorylation of TYRO3, AXL and MERTK
22 (Supplementary Figure S6). Pharmacological inhibition of TAM receptors with UNC-2025
23 and BMS-777607 significantly decreased in a dose-dependent manner the viability of
24 RT112, MGH-U3, VM-CUB-1 and UM-UC-5 cells, whereas UM-UC-9 cells were more

1 resistant (Figure 2C and Supplementary Table S3). In line with our TAM receptor
2 depletion experimental results (Figure 2B), these effects appeared to be more strongly
3 linked to TYRO3 inhibition. Indeed, MGH-U3 cells, which express only TYRO3, and
4 RT112, which do not express MERTK and are resistant to AXL silencing, were not only
5 the most sensitive cell lines to TYRO3 depletion but also to the TAM receptor inhibitors.
6 Although we cannot rule out that a double or a triple knockdown could have a greater
7 impact on cell viability than a single knockdown, we did not observe a greater sensitivity
8 of VM-CUB-1, which expresses TYRO3, AXL and MERTK, to the pan-TAM inhibitors
9 than the cell lines expressing TYRO3 only or in combination with a second TAM
10 receptor. In the other hand, we could not exclude that MET or FLT3 inhibition
11 contributed also to the observed phenotype since the cells express both receptors (data
12 not shown).

14 ***TYRO3* knockdown reduces anchorage-independent cell growth *in vitro* and tumor** 15 **growth *in vivo***

16 We then investigated whether this identified role of TYRO3 in bladder cancer cells was
17 relevant to clonogenic cells, by analyzing the consequences of *TYRO3* knockdown for
18 the anchorage-independent growth of TYRO3-dependent bladder cancer cell lines *in*
19 *vitro* and tumor growth *in vivo*. *TYRO3* depletion significantly decreased the number of
20 viable MGH-U3 and RT112 colonies in soft agar assays, by 80% and 60%, respectively,
21 demonstrating a role for TYRO3 in regulating the survival of clonogenic cancer cells
22 (Figure 3A).

23 We also found that the treatment of established MGH-U3 xenografts in nude mice with
24 intraperitoneal injections of *TYRO3* siRNA reduced significantly the tumor growth as

1 compared to in similar tumors treated with a scramble siRNA ($\approx 354 \text{ mm}^3$ versus 1090
2 mm^3 at day 21, $p < 0.0001$) (Figure 3B). This inhibition of tumor growth was associated
3 with significantly lower *TYRO3* mRNA levels on RT-qPCR (Figure 3C).

4 These results indicate that the genetic depletion and pharmaceutical inhibition of *TYRO3*
5 lead to decreases in bladder cancer cell viability and tumor outgrowth ability, identifying
6 *TYRO3* as a potential therapeutic target.

7 8 ***TYRO3* depletion induces tumor suppressor pathways and apoptosis, while** 9 **inhibiting proliferative pathways**

10 We investigated the molecular mechanisms underlying the oncogenic dependency on
11 *TYRO3* expression in bladder carcinomas, by conducting a comprehensive gene
12 expression analysis with Affymetrix U133 Plus 2.0 arrays, in MGH-U3, RT112 and UM-
13 UC-5 cells transfected with three different *TYRO3* siRNAs. We identified 284 genes as
14 differentially expressed after *TYRO3* knockdown in these three cell lines (adjusted p -
15 value < 0.05 , $|\log_2(\text{FC})| \geq 0.58$ (see methods); supplementary table S4). An analysis of
16 this list of genes with Ingenuity Pathway Analysis (IPA) software identified key molecular
17 and cellular functions (Figure 4A, upper panel) and upstream regulators/ transcription
18 factors presenting significant alterations upon *TYRO3* knockdown (Figure 4A, lower
19 panel). *TYRO3*-regulated genes were highly significantly enriched in gene sets involved
20 in the “cell cycle”, “cell proliferation”, “cell survival”, “cell apoptosis” and “cell death”
21 functions (Figure 4A upper panel). The gene sets corresponding to each function are
22 listed in supplementary table S5. Consistent with these findings, cell cycle and cell
23 survival processes were significantly downregulated (negative z -score), whereas cell

1 death and apoptosis, in particular, were upregulated (positive z-score) upon *TYRO3*
2 knockdown (Figure 4A, upper panel). We also identified, among the top ten
3 transcriptional regulators whose activities were predicted to be upregulated or
4 downregulated by *TYRO3* depletion, genes encoding important regulators of the cell
5 cycle and cell survival such as *MYC*, *FOXM1*, *E2F1*, *E2F2*, *E2F3* and *CCND1*, whose
6 activity were predicted to be downregulated whereas the activities of the tumor
7 suppressor transcription factors *TP53* and *RB1* were predicted to be upregulated (Figure
8 4A, lower panel, table S6). Strikingly, *CDKN2A* was also predicted as up-regulated upon
9 *TYRO3* depletion whereas cell lines harbor a *CDKN2A* homozygous gene deletion²¹
10 highlighting the need for prediction validation that can be biased if targeted genes used
11 for a transcription regulator are also controlled by other regulators. Western-blot analysis
12 showed a downregulation of *FOXM1* and *CYCLIN D1* levels following *TYRO3* depletion
13 in MGH-U3, RT112 and UM-UC-5 cells, potentially accounting for the lower levels of
14 activity for these transcription factors highlighted by our transcriptomic data analysis in
15 these cell lines (Figure 4B and Figure 4A, lower panel). Although IPA predicted an
16 inhibition of *MYC*, western-blot analysis showed a decrease in c-MYC protein levels
17 mainly in UM-UC-5 and RT112. Conversely, during cell cycle progression,
18 retinoblastoma protein (pRB) is sequentially phosphorylated by *CYCLIN-CDK*
19 complexes, and *CYCLIN D1-CDK4* specifically phosphorylates pRB on the Ser780
20 residue, leading to its inactivation and the release of *E2F*²². In accordance with our
21 transcriptomic data showing that both *CCND1* and *CDK4* gene expression were
22 downregulated upon *TYRO3* depletion (supplementary table S4), we observed lower
23 levels of pRB phosphorylation at Ser780 in the three cell lines studied after *TYRO3*
24 knockdown, which may, therefore, lead to pRB activation and *E2F* inactivation, as

suggested by IPA analysis of our transcriptomic data (Figure 4B and Figure 4A, lower panel). Western-blot analysis also confirmed, at the protein level, the downregulation of the AURORA A and AURORA B kinases involved in cell cycle entry and mitotic spindle assembly, and of SURVIVIN, for which mRNA levels had already been shown to be decreased by *TYRO3* depletion ($\log_2FC=-1.104$; -1.159 and -1.298 , respectively) (Figure 4B). Taken together, our data for the MGH-U3, RT112 and UM-UC-5 cell lines demonstrate that *TYRO3* depletion inhibits proliferative pathways and activates tumor suppressor pathways. These molecular mechanisms are undoubtedly of general importance, because all our western-blot results (Figure 4B) were confirmed in two other *TYRO3*-dependent cell lines, VM-CUB-1 and UM-UC-9 (Supplementary Figure S7). Given that the pRB pathway is frequently altered in bladder cancer, particularly through direct *RB1* gene inactivation, we assessed the impact of *TYRO3* depletion in the *RB1* mutated bladder cancer cell lines, 5637 (pY325*), 647V (p.Q383*) and HT1376 (p.Q383*)¹⁷. The cell viability of these *RB1* mutant cells was significantly decreased upon *TYRO3* silencing (Supplementary Figure S8). Although the impact of *TYRO3* depletion was not as important as the one observed in *RB1* wild type cells, these results suggest that the sensitivity to *TYRO3* inhibition is not strictly dependent on *RB1* gene status.

SURVIVIN is not only involved in the cell cycle, in which it regulates the mitotic spindle checkpoint, but also in the inhibition of caspase-9, an initiator caspase involved in caspase-3 activation²³. Its loss is, thus, consistent with the induction of apoptosis upon *TYRO3* silencing, through caspase activation (Figure 4A, upper panel). Western-blot analysis on lysates of *TYRO3* siRNA-transfected RT112 cells showed a greater accumulation, over time, of the cleaved forms of the effector caspase-3 and the initiator

1 caspase-8, resulting in the cleavage of PARP, one of the main targets of caspase-3,
2 thereby clearly demonstrating the activation of apoptosis by *TYRO3* depletion (Figure
3 4C, left panel). These results were confirmed 72 h after transfection with *TYRO3* siRNA,
4 in MGH-U3 and UM-UC-5 cells (Figure 4C, right panel), and cleaved PARP was also
5 identified in VM-CUB-1 and UM-UC-9 cells after *TYRO3* knockdown (Supplementary
6 Figure S7). Moreover, inhibitors of either caspase-3/7 (z-IEVD-fmk) or caspase-8 (z-
7 IETD-fmk) significantly reduced the impact of *TYRO3* depletion on cell survival,
8 demonstrating the crucial importance of apoptosis regulation for *TYRO3* activity in
9 bladder cancer cells (Figure 4D). Finally, a TUNEL assay performed in our MGH-U3
10 xenografted mouse model (Figure 3) clearly showed that apoptosis levels were
11 significantly higher in *TYRO3* siRNA-treated animals (Figure 4E).

13 **DISCUSSION**

14 One of the major aims of this study was to assess the expression of TAM receptors in
15 bladder cancer. Our study showed that only *TYRO3* expression levels were significantly
16 increased in both NMIBCs and MIBCs relative to normal urothelium samples. The
17 aberrant expression of TAM receptors is frequently observed in several cancers and has
18 been associated with aggressive cancer phenotypes, overall poor patient survival and
19 the emergence of drug resistance⁹. *TYRO3* has been less studied than the other two
20 TAM receptors, but several studies have reported results similar to ours, highlighting the
21 contribution of this receptor in various types of cancer, leiomyosarcoma, melanoma,
22 colorectal and thyroid cancers^{18,24–27}. However, *TYRO3* expression in bladder cancer is
23 not correlated with disease stage (contrary to reports for colon cancer), suggesting a
24 possible role for *TYRO3* in the formation of bladder cancers, as well as in their

1 progression. However, we can assume that TYRO3 plays a greater role in MIBCs than
2 in NMIBCs, due to the stronger expression of its ligand, GAS6, in MIBCs than in
3 NMIBCs. Nevertheless, ligand-independent activation has also been reported for strong
4 TYRO3 expression ⁶, and we show here that TYRO3 is N-glycosylated in bladder
5 tumors, which might lead to its ligand-independent activation in the presence of galectin,
6 in a process similar to that described for N-glycosylated vascular endothelial growth
7 factor receptor 2, which is activated by galectin-1 in the absence of ligand ²⁸.

8 No alteration of the *TYRO3* copy number was detected (data not shown), and the
9 mechanisms increasing the expression of TYRO3 in bladder tumor cells remain to be
10 determined. TYRO3 was detected at the plasma membrane in several bladder cancer
11 cell lines by flow cytometry, but IHC staining on human bladder tumor sections showed it
12 to be present mostly in the cytoplasm and only weakly expressed at the plasma
13 membrane of tumor cells. A similar RTK distribution has already been reported for
14 TYRO3 and AXL in human colorectal cancer and pancreatic cancer, respectively ^{18,29}.

15
16 Consistent with this general higher level of expression of *TYRO3* in bladder tumors
17 compared to normal samples, TYRO3 expression was detected in most of the UBC cell
18 lines, whereas AXL and MERTK had more heterogeneous expression patterns in UBC
19 cell lines. *TYRO3* knockdown systematically resulted in a significant large decrease in
20 cell growth/survival, in all the bladder cancer cell lines tested, regardless of AXL and
21 MERTK levels, as previously reported for melanoma cells ³⁰. These results suggest that
22 neither AXL nor MERTK could play a redundant role, rescuing the loss of TYRO3. The
23 three TAM receptors therefore have different roles in bladder tumors. Accordingly, the
24 impact of *TYRO3* knockdown on UBC cell proliferation was consistently greater than the

1 impact of *MERTK* or *AXL* knockdown. Despite the preponderant role of *TYRO3* in the
2 control of cancer cell growth/survival, we cannot rule out the possibility that *AXL* or
3 *MERTK* knockdown affects other processes known to be regulated by TAM receptors,
4 such as cell migration, and that long term inhibition of *AXL* or *MERTK* could impact
5 tumor cell viability ³¹.

6
7 Our transcriptomic analysis, with validation by immunoblotting, showed that *TYRO3*
8 silencing inhibited the cell cycle and cell survival processes, whilst inducing apoptosis.
9 Such effects of *TYRO3* knockdown have already been reported in other cancer types:
10 melanoma, thyroid and breast cancer cells ^{25,26,32}. In breast cancer cells, as reported
11 here for UBC cells, the regulation of these processes may involve a decrease in *CYCLIN*
12 *D1* and *SURVIVIN* levels induced by *TYRO3* knockdown ³². Our transcriptomic analysis
13 suggested a role for *MITF* regulation, as previously reported for melanoma ²⁶, but the
14 involvement of *MITF* in bladder tumors has never been explored. Other pathways were
15 identified as regulated in both studies, including proliferation, survival/growth and
16 apoptosis, suggesting possible roles for *TYRO3* in these processes and the existence of
17 general features relating to *TYRO3* activity in different cancer types. However, some
18 cancer type-specific features may fine-tune *TYRO3*-mediated signaling pathways and
19 their outcomes. Indeed, *TYRO3* has been shown to stimulate not only cell proliferation,
20 but also cell migration and EMT processes, via the regulation of *SNAI1*, in colorectal
21 cancer ¹⁸, whereas our transcriptomic analysis detected no deregulation of cell migration
22 or EMT processes following *TYRO3* knockdown in three different UBC cell lines.

23 In addition to this cell-autonomous effect in bladder cancer, *TYRO3* may also have cell-
24 non autonomous effects. Indeed, TAM receptors have been shown to favor the escape

of tumor cells from immune surveillance by activating innate immune checkpoints and, in particular, by upregulating PD-L1 expression^{33,34}. Our transcriptomic analysis upon *TYRO3* depletion revealed no modulation of *CD274* (PD-L1) expression, but a significant increase in *TNFSF9* (CD137L) expression. Given the current interest in agonistic anti-CD137 antibodies for cancer treatment³⁵, *TYRO3* inhibition might be useful for both inhibiting tumor growth and boosting the antitumor immune response. In line with this hypothesis, a phase 1b clinical trial (NCT03170960) has recently begun to test the combination of a pan-TAM inhibitor (cabozantinib) with an anti-PD-L1 antibody (atezolizumab) in patients with advanced urothelial carcinoma (including bladder, renal pelvis, ureter, or urethra carcinoma) or renal cell carcinoma.

Overall, our data suggest that *TYRO3* is a promising potential therapeutic target for both muscle-invasive and non-muscle invasive bladder cancers. Although our pharmaceutical inhibition experiments with TAM inhibitors reproduced the results obtained with TAM knockdown, we cannot rule out the possibility that other activities of UNC-2025 or BMS-777607 could impact tumor cell viability. Given the preclinical evidence of the involvement of *TYRO3* in cancer, it is important to develop potent small inhibitors or monoclonal antibodies specific against this tyrosine kinase receptor. Efforts in such direction are being made^{18,30}.

AUTHORS' CONTRIBUTIONS

Conception and design: IBP, FR, and FD

Development of methodology: IBP, YL, VD, HH, HNK and TM

Acquisition of data: FD, HNK, LS, AMV, CK, NK, MDG, PM, EC, NS and IBP

1 Analysis and interpretation of data: IBP, FD, EC, VD, HH, TM, NS and YA
2 Writing, review, and/or revision of the manuscript: IBP, FD, FR, YA and VD
3 Study supervision: IBP, FR and VD
4

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20

21 **CONFLICT OF INTEREST**

22 IBP, YA, NS and FR hold a patent describing TYRO3 as a therapeutic target for the
23 treatment of cancer (patent publication WO/2010/031828), which has been licensed to
24 ElsaLys Biotech. FD, HH, TM, VD, FR and IBP hold a patent describing anti-TYRO3

antibodies (patent publication WO/2016/166348). The other authors have no potential conflicts of interest to disclose.

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12 **FIGURE LEGENDS**

13 **Figure 1: TYRO3 is highly expressed in bladder cancer and is N-glycosylated**

14 A. *TYRO3*, *AXL*, *MERTK* and *GAS6* mRNA levels, as measured by RT-qPCR, in the CIT
15 cohort of non-muscle-invasive bladder cancers (NMIBCs, $n = 87$) and muscle-invasive
16 bladder cancers (MIBCs, $n = 82$) and in normal urothelium (NU, $n = 7$). The significance
17 of differences was assessed in Mann-Whitney tests, and the means and standard errors
18 are shown.

19 B. Lysates from a subset of human tumors from the CIT cohort and from control normal
20 urothelium (NU) and normal vesical muscle (NM) were analyzed by western blotting with
21 antibodies targeting the indicated proteins. NMIBCs and MIBCs were identified by one
22 and two asterisks, respectively.

23 C. TYRO3 immunoblots for tumor lysates (CIT.56 and CIT.190) treated with peptide-N-
24 glycosidase F (PNGase F).

D. Representative images of TYRO3 immuno-histochemical staining for a panel of human bladder tumors (NMIBC and MIBC) and urothelium from histologically normal tissue adjacent to the tumor (NAT). Black bars represent 100 μ m. IHC validation experiments are available in supplementary figure S3.

Figure 2: TAM knockdown and pharmaceutical inhibition studies in bladder cancer cell lines reveal the major contribution of TYRO3 to cell viability

A. Lysates from a panel of human urothelial bladder cancer cell lines were analyzed by western blotting for TAM expression. Alpha tubulin served as a loading control.

B. Knockdown of *TYRO3*, *AXL* and *MERTK*, with three different siRNAs for each receptor, in a panel of bladder cancer cell lines. Cell survival was quantified by CellTiter-Glo at 72 h and 96 h. The TAM receptor(s) that is (are) expressed in each cell line is (are) indicated beneath their name.

C. Dose-response curves for bladder cancer cells treated with BMS-777607 and UNC-2025, two pan-TAM inhibitors. Cell viability was quantified by CellTiter-Glo at 72h.

The data shown are the means of three independent experiments, and error bars represent the SD. $*p < 0.05$. Statistical analyses based on unpaired *t*-tests with Welch's correction are shown in supplementary tables S2 and S3.

Figure 3: *TYRO3* depletion decreases colony-forming potential and tumorigenesis

A. Impact of *TYRO3* knockdown on the anchorage-independent growth of MGH-U3 and RT112 cells. Colonies in soft agar with a diameter larger than 50 μ m were quantified 14 days after transfection with a control siRNA (siCTL) or with *TYRO3* siRNA#1. Three experiments were realized and the results shown are from a representative experiment

1 conducted in triplicate. The data shown are means $\pm$ SD. Unpaired *t*-tests were used for
2 statistical comparison.

3 B. MGH-U3 bladder cancer cells were injected subcutaneously into nude mice (*n*=6
4 animals/group, two xenografts per animal (one in each flank)). When tumor volume
5 reached 50($\pm$ 5) mm³, tumor-bearing mice were treated three times weekly with
6 intraperitoneal injections of 4 μ g control scramble siRNA or *TYRO3* siRNA#1 (the first
7 injection corresponds to day 0). Tumor volumes were measured at the indicated time
8 points and tumor volumes were calculated. Data are presented as means $\pm$ SEM. Tumor
9 volumes were compared in Wilcoxon's tests.

10 C. MGH-U3-derived xenograft tumors from mice treated with scramble siRNA or *TYRO3*
11 siRNA#1 were lysed and *TYRO3* expression was measured by RT-qPCR. *TBP* was
12 used as housekeeping gene. The data shown are means $\pm$ SD of two independent
13 experiments performed in triplicate. Statistical analysis was performed with unpaired *t*-
14 tests.

15 **Figure 4: *TYRO3* silencing inhibits the cell cycle and cell proliferation and induces**
16 **apoptosis**

17 A. Transcriptomic analysis upon *TYRO3* knockdown in MGH-U3, RT112 and UM-UC-5
18 cells. Ingenuity Pathway Analysis (IPA) was performed for a list of 284 genes
19 differentially expressed upon *TYRO3* silencing (fold-change $|\log_2| \geq 0.58$ and a *p*-value
20 < 0.05). Upper panel: Histograms showing the cellular functions predicted by IPA to be
21 deregulated after *TYRO3* knockdown. *p*-values (hatched bar), and negative (white bar)
22 and positive (black bar) absolute z-scores are indicated. Lower panel: Diagram showing
23 the upstream regulators (transcription factors) significantly predicted by IPA to be

involved in the regulation of gene expression observed after *TYRO3* knockdown (p -value $< 1E-6$).

B-C. Western-blot analysis of *TYRO3*, *AXL*, *MERTK*, *FOXM1*, *c-MYC*, *CYCLIN D1*, phospho-pRB (P-pRB S780) and total pRB, *AURORA A* and *B*, *SURVIVIN* levels in total cell lysates from MGH-U3, RT112 and UM-UC-5 cells transfected with a control siRNA (siCTL) or with *TYRO3* siRNAs (siTYRO3#1, siTYRO3#2 and siTYRO3#3) for 72h. (C) The activation of apoptosis was assessed at the indicated period of time by monitoring cleaved PARP, caspase-8 (CASP8) levels, with detection of the proform (CASP8) and cleaved form (p43/41), and caspase-3 (CASP3). Western blot results are representative of at least two independent experiments.

D. MGH-U3 and RT112 cells were transfected with either a control siRNA (siCTL) or with *TYRO3* siRNA#1 for 24 h. They were then treated with DMSO, 50 μ M Z-IETD-FMK (caspase-8 inhibitor) and 50 μ M Z-DEVD-FMK (caspase-3/7 inhibitor) for 48 h. Cell viability was assessed by measuring MTT incorporation at 72 h. The results are expressed as the means $\pm$ SD of three experiments. An unpaired t -test was used for statistical analysis.

E. Sections of MGH-U3-derived xenograft tumors from mice treated with scramble siRNA or *TYRO3* siRNA#1 were stained for DNA fragmentation with a TUNEL assay detection kit ($n=4$ mice per group). Representative sections are presented (left panel, black bar = 100 μ m) and the percentage of cells displaying apoptosis (right panel) was evaluated. The data shown are means $\pm$ SD ($n=4$). Statistical analysis was performed with t -tests.

Supplementary Figure S1: TAM receptors and GAS6 expression in bladder cancer tumors from the TCGA cohort

Box plot of *TYRO3*, *AXL*, *MERTK* and *GAS6* expression levels measured by IlluminaHiSeq_RNASeq in MIBCs (tumors, $n = 405$) and peritumoral bladder tissue with a normal histological appearance (normal tissues, $n = 14$) from the TCGA cohort. From the 19 normal tissues available in the TCGA cohort, we selected the 14 samples expressing UPK2, demonstrating the presence of urothelium in these samples. RPKM = reads per kilobase per million mapped reads. Plots show means $\pm$ SD. Statistical analysis was performed with Mann-Whitney tests.

Supplementary Figure S2: Correlation between TAM receptors mRNA and protein levels in the CIT cohort

Quantification of TAM receptors protein expression from western-blot gels (for each receptor one blot encompassing 25 samples presented in Figure 1B and a second blot encompassing 18 samples (data not shown) were quantified) Relative protein levels were calculated by dividing the signal intensity for each TAM receptors by the corresponding tubulin intensity. For each TAM receptor, mRNA and corresponding protein level were plotted for the different samples ($n=43$). Spearman's coefficient and P -values are indicated for each TAM receptors.

Supplementary Figure S3: Specificity of the anti-TYRO3 antibody used in the immunohistochemistry experiments

The specificity of the anti-TYRO3 antibody used in the immunohistochemistry experiments (HPA071245, Sigma-Aldrich) was assessed in 5637 cells, a bladder cancer

cell line expressing TYRO3, AXL and MERTK, transfected with control siRNA (siCTL) or *TYRO3* siRNA#2 for 48 h. Some of the cells were lysed in Laemmli lysis buffer for TAM receptors western-blot analysis (A), the rest of the cells were pelleted, fixed in formalin, embedded in paraffin and used for immunohistochemistry to assess TYRO3 expression (B).

Supplementary Figure S4: TAM knockdown in bladder cancer cells

Knockdown of *TYRO3*, *AXL* and *MERTK* with three different siRNAs in the bladder cancer cell lines used in Figure 2B. The TAM receptor(s) which is(are) expressed in each cell line is indicated below the name of each model. Cells were collected and lysed after 72 h. Cell lysates were analyzed by western blotting for TAM receptors, with beta-actin as a loading control.

Supplementary Figure S5: TYRO3 is expressed at the surface of MGH-U3, UM-UC-5 and RT112 cells

A. Validation of TYRO3 staining by flow cytometry, for UM-UC-5 cells transfected with control siRNA (siCTL) or *TYRO3* siRNA (siTYRO3#1). Gray filled histogram, isotype control. Unfilled/green-line histograms, TYRO3 staining.

B. Flow cytometry analysis of TYRO3 expression in three human bladder cancer cell lines: MGH-U3, UM-UC-5 and RT112. Gray filled histogram, isotype control. Unfilled/green-line histograms, TYRO3 staining.

In these experiments, we used the anti-TYRO3 antibody clone 5B4 (Ref: GTX83459) from GeneTex .

Supplementary Figure S6: Impact of BMS-777607 and UNC-2025 on TAM receptors phosphorylation

VM-CUB-1 cells were treated for one hour without inhibitor (control DMSO), with BMS-777607 (5 μ M) or UNC-2025 (5 μ M). Phospho-tyrosines were immunoprecipitated using PY20 antibody and TYRO3, MERTK and AXL protein content in the immunoprecipitates were analyzed by western blotting.

Supplementary Figure S7: *TYRO3* depletion decreases the levels of proteins involved in cell cycle/proliferation control and triggers apoptosis in VM-CUB-1 and UM-UC-9 cells

Western-blot analysis of TYRO3, AXL, MERTK, FOXM1, c-MYC, CYCLIN D1, phospho-pRB (P-pRB S780) and total pRB, AURORA A and B, SURVIVIN, cleaved PARP levels in total cell lysates from VM-CUB-1 and UM-UC-9 cells transfected with a control siRNA (siCTL) or with three different *TYRO3* siRNAs (siTYRO3#1, siTYRO3#2 and siTYRO3#3) for 72 h.

Supplementary Figure S8: *TYRO3* depletion in RB1 mutant bladder cancer cell lines

The RB1 mutant bladder cancer cell lines 5637 (RB1 p.Y325*), 647V (RB1 p.Q383*) and HT1376 (RB1 p.Q702*) were transfected with a control siRNA (siCTL) or with three different *TYRO3* siRNAs (siTYRO3#1, siTYRO3#2 and siTYRO3#3). Western-blot analyses of TAM receptors were carried out on total cell lysates after 72h. Cell survival was quantified by CellTiter-Glo at 72 h.