Tumor Suppressor RARRES1 Interacts with Cytoplasmic Carboxypeptidase AGBL2 to Regulate the α-Tubulin Tyrosination Cycle

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Abstract

Even though it is among the most commonly methylated loci in multiple cancers, the retinoic acid–induced tumor suppressor retinoic acid receptor responder 1 (RARRES1) has no known function. We now show that RARRES1 is lost in many cancer cells, particularly those with a mesenchymal phenotype, and is a transmembrane carboxypeptidase inhibitor that interacts with ATP/GTP binding protein-like 2 (AGBL2), a cytoplasmic carboxypeptidase. Knockdown of AGBL2 results in a failure of the cell to detyrosinate the C-terminal EEY region of α-tubulin and indicates that it is a candidate for the long sought-after tubulin tyrosine carboxypeptidase important in the regulation of microtubule dynamics. In contrast, knockdown of RARRES1 increases the level of detyrosinated α-tubulin consistent with a role as the cognate inhibitor of AGBL2. We conclude that RARRES1, its interacting partners AGBL2, Eg5/KIF11, another EEY-bearing protein (EB1), and the microtubule tyrosination cycle are important in tumorigenesis and identify a novel area for therapeutic intervention.

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Introduction

Retinoic acid receptor responder 1 (RARRES1), also known as tazarotene-induced gene 1, was first identified as a novel retinoid-responsive gene in skin. It is induced in a retinoic acid receptor–specific manner in a variety of human skin-related systems (1). The RARRES1 promoter region is methylated in primary prostate cancers compared with normal tissues or benign hyperplasias, and its decreased expression is associated with an increase in the malignant potential of prostate carcinoma cells (2). RARRES1 is among the most commonly methylated loci in multiple cancers and is often described as a putative tumor suppressor gene (3). In addition, RARRES1 also plays a role in the proliferative/differentiative switch in adult adipose-derived mesenchymal stem cells. Notably, a RARRES1 family member, latexin (LXN), has been implicated in controlling aspects of stem cell biology, in which an inverse and direct relationship between LXN expression and the size of the hematopoietic stem cell population in mice has been reported (4). LXN was initially described as the only known mammalian carboxypeptidase inhibitor and is involved in the regional specification of neurons (5–8). Despite extensive evidence for a tumor suppressor role of RARRES1, no mechanism for its biological function has been determined.

Here, we show that RARRES1 is a type III membrane protein found in a complex with several proteins involved in the regulation of microtubule function and reveal that ATP/GTP binding protein-like 2 (AGBL2; a carboxypeptidase) and RARRES1 (an inhibitor) regulate the tubulin tyrosination cycle. Our findings point to a role for RARRES1, AGBL2, and the microtubule tyrosination cycle in cancer and identify a novel avenue for potential therapeutic intervention.

Materials and Methods

Cells

Immortalized human prostate epithelial PWR-1E cells (a gift from Dr. S.C. Chauhan, University of South Dakota), PC-3 human prostate cancer cells, SKBR-3 human breast cancer cells, and HEK 293 human embryonic kidney cells were maintained according to the recommendation of American Type Culture Collection.

Antibodies and reagents

Primary antibodies targeting the following antigens were used: goat anti-human RARRES1 (catalogue no. AF4255, R&D Systems), antityrosinated tubulin, and anti-Eg5 (Abcam),
anti-E-cadherin (BD-Transduction Laboratories), anti-cyclin D1 (EMD-Calbiochem), anti-β-catenin (Millipore), anti-histone H4 (Cell Signaling), anti-detyrosinated tubulin (AbD Serotec), and anti-AGBL2, and anti-pan-cadherin (Sigma-Aldrich). The following inhibitory RNAs (RNAi; Dharmaco) were used: for RARRES1 knockdown, GUAACGCCGUACUGGAAA and AAAGAGGGAGUAAGGUC. For AGBL2 knockdown: GCA-CACUUCCACCCAUUA and UGGACAGAGUAGAUUUA.

siRNA, expression constructs, and transfection

Variant and full-length human RARRES1 isoforms were directionally cloned into the Bgl II and HindIII sites in the pEYFP-N1 vector (Clontech). N-RARRES1 (+121 to +897) and full-length RARRES1 (+1 to +897) were cloned into the pGlu vector as codon-optimized versions by Genscript. AGBL2 (catalogue no. M-012937-00) and nontargeting control siRNAs (catalogue no. D-001210-01-20) were from Dharmaco. Plasmid DNA and siRNA constructs were introduced to cells either by electroporation mediated by an Amaxa nucleoporator (Amaza-Lonza) or by Fugene 6 transfection reagent (Roche).

Detection of AGBL family members by quantitative PCR

Total RNA was extracted from indicated cell lines with Trizol reagent (Invitrogen) and isolated with an RNAeasy purification kit (Qiagen). Single-stranded cDNA was prepared from 400 ng of RNA by TaqMan reverse transcription reagents (Applied Biosystems). Real-time PCR (RT-PCR) was then carried out and monitored on a 7900 HT system (Applied Biosystems), using TaqMan universal PCR master mix and the following inventoried primer/probe sets: Hs00328701_m1, AGBL3–Hs00417079_m1, AGBL1/Nna1–Hs00262179_m1, and AGBL5–Hs00222447_m1 (Applied Biosystems). Plotted ΔCt values were determined by subtracting control [glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] cycle threshold values from each target cycle threshold. Where targets did not return a threshold value after 40 cycles of PCR, the transcript was determined to be absent.

RARRES1 RT-PCR

RARRES1 (full-length) forward 5′-CAACAAGGAGGATCCTGCTTTAACAAG-3′ and reverse 5′-GAGCCAGAGTTCGTTGAGC-3′ primers (generating a 630-base pair amplicon). For β-actin, forward 5′-CCACTGCGCATGCGCATG-3′ and reverse 5′-GCGGATGGTCAGCTCAGCTCACT-3′ primers (generating a 350-base pair amplicon). Thermal cycling for RARRES1 was done according to the following profile: 30 minutes at 50°C, 15 minutes at 95°C for the reverse transcriptase reaction, followed by PCR cycling of 30 seconds at 94°C, 45 seconds at 52°C, 1 minute at 72°C, for indicated number of cycles with a subsequent final elongation at 72°C for 10 minutes.

PNGase F digestion

Whole cell lysates of untreated PWR-1E cells were made with RIPA buffer, followed by digestion with the glycosidase PNGase F, or no enzyme (NEB), according to the manufacturer’s instructions.

Cell fractionation

Three-pool (membrane, cytoplasmic, and nuclear/cell debris fractions) cell fractionation was carried out as previously described (9).

Cell lysis and tandem affinity purification

A stable clone expressing a level of exogenous pGlu RARRES1 close to the endogenous level of this protein was grown in 5 dishes (150 mm each). At 90% confluence, medium was discarded and each dish was lysed in 0.5 mL of a lysis buffer, composed of 10% glycerol, 50 mmol/L HEPES-NaOH, pH 8.0, 100 mmol/L NaCl, 2 mmol/L EDTA, 0.1% NP-40, 2 mmol/L DTT, 10 mmol/L NaF, 5 mmol/L calyculin A, 50 mmol/L β-glycerolphosphate, and 1× Complete Mini protease inhibitor (Roche). Lysates were harvested by scraping and 2 freeze-thaw cycles were done to improve protein recovery. Lysates were then centrifuged at 15,000 × g for 15 minutes, and supernatant were recovered and incubated for 4 hours with 100 μL of streptavidin beads (Streptavidin Sepharose High Performance, GE Healthcare) prewashed 3 times with the lysis buffer. The slurry was then centrifuged at 1,500 × g for 2 minutes, and the precipitate containing the streptavidin beads was recovered and washed 3 times with lysis buffer and 2 times with the TEV buffer, supplied with the AcTEV Protease kit (catalogue no. 12575-015; Invitrogen). Streptavidin beads were then incubated with 200 units of TEV protease in 150 μL of TEV buffer overnight at 4°C. The slurry was then centrifuged at 1,500 × g for 2 minutes, and the supernatant was recovered. The precipitated beads were washed twice with 200 μL of TEV buffer, centrifuged, and supernatants were pooled. The final pool volume was diluted 1:1 (v/v) with calmodulin-binding buffer composed of 10 mmol/L β-mercaptoethanol, 10 mmol/L HEPES-NaOH, pH 8.0, 150 mmol/L NaCl, 1 mmol/L MgOAc, 1 mmol/L imidazole, 0.1% NP-40, and 2 mmol/L CaCl2. The mixture was then incubated for 90 minutes at 4°C with 100 μL of calmodulin beads (Calmodulin Sepharose 4B; GE Healthcare) prewashed 3 times with calmodulin-binding buffer. The slurry was then centrifuged at 1,500 × g for 2 minutes, the supernatant was discarded, and the precipitated beads were washed twice with a calmodulin-rinsing buffer composed of 50 mmol/L ammonium bicarbonate, pH 8.0, 75 mmol/L NaCl, 1 mmol/L MgOAc, 1 mmol/L imidazole, and 2 mmol/L CaCl2. Each wash was followed by centrifugation and supernatant shedding. A total of 150 μL of a calmodulin-elution buffer composed of 50 mmol/L ammonium bicarbonate, pH 8.0, and 25 mmol/L EGTA was then added to the calmodulin resin. The slurry was then vortex mixed, centrifuged at 1,500 × g, and the supernatant was collected. This elution step was repeated twice and supernatants were pooled together (Supplementary Fig. S1).

Trypsin digestion and liquid chromatography/tandem mass spectrometric analysis

The pull-down sample was vacuum-dried and reconstituted with 20 μL of a buffer composed of 500 mmol/L triethylammonium bicarbonate, pH 8.5. The protein sample was then denatured by adding 1 μL of a 2% SDS solution followed by the addition of 2 μL of a reducing reagent composed of 50 mmol/L...
TRIS-(2-carboxyethyl)phosphine. The mixture was then incubated at 60 °C for 1 hour followed by the addition of 1 μL of a cysteine-blocking reagent composed of 200 mmol/L methyl methane-thiosulfonate (MMTS) in isopropanol. Trypsin digestion was then done by adding 10 μL of 1 μg/μL trypsin solution in 80 mmol/L CaCl₂. Samples were incubated overnight at 37 °C, vacuum dried, and then reconstituted with 10 μL of a 2% acetonitrile and 0.1% formic acid in distilled deionized water, as described previously (10). Nano liquid chromatography/tandem mass spectrometry (LC/MS-MS) was done using a Q-Star Elite (Applied Biosystems), equipped with a nanoAcquity UPLC system (Waters). Sample separations were done with a 1.7-μm nanoAcquity BEH130 C18 column (100 μm × 100 mm) at a flow rate of 400 nL/min. Tryptic digests were eluted by the following gradient: 100% of solvent A [97.9% water, 2% acetonitrile, 0.1% formic acid (v/v/v)] for 1 hour; then from 100% solvent A to 100% solvent B [2% water, 97.8% acetonitrile, 0.1% formic acid (v/v/v)] in 2 hours; finally, A 100% solvent B flow was maintained for 1 hour, followed by a return to 100% of solvent A flow in 15 minutes. Mass spectrometric settings were as follows: ion spray voltage, 2,300 V; interface heater temperature, 220 °C; cone voltage, 20 V; and collision energy, 8 V.

**Protein identification**

Protein identification was done using ProteinPilot software with the following settings (11): sample type: identification; cysteine alkylation: MMTS; digestion: trypsin; instrument: QSTAR ESI; species: Homo sapiens; cysteine alkylation: MMTS; digestion: trypsin; instrument: QSTAR ESI; species: Homo sapiens; MS/MS fragment tolerance of 0.3 Da.

**Western blotting**

Western blotting was done as described previously (12, 13). Cells were lysed with RIPA buffer and loaded onto 4% to 12% gradient polyacrylamide gel. Amounts of proteins loaded were calculated at 60 μg of total cell lysates for tubulin immunoblots and 10 μg for all other immunoblots. Gels were electrophoresed at 100 V until the end of the separation. Proteins contained within the gel were then electroblotted onto a nitrocellulose membrane (50 V for 50 minutes). Western blot analyses were accomplished by a 1 μg/mL dilution of primary antibody, followed by incubation with a horseradish peroxidase–conjugated secondary antibody against the appropriate species. Visualization of the bands was then accomplished by the addition of a 1:1 ratio of Super Signal West Pico-Stable Peroxidase Solution and Luminol/Enhancer Solution (Pierce) and by developing the chemiluminescent signal in the dark with the Kodak Scientific Luminol/Enhancer Solution (Pierce) and by developing the chemiluminescent signal in the dark with the Kodak Scientific Luminol/Enhancer Solution (Pierce) and by developing the chemiluminescent signal in the dark with the Kodak Scientific Luminol/Enhancer Solution (Pierce) and by developing the chemiluminescent signal in the dark with the Kodak Scientific Luminol/Enhancer Solution (Pierce) and by developing the chemiluminescent signal in the dark with the Kodak Scientific Luminol/Enhancer Solution (Pierce). Consistent laser intensity or camera exposure levels for each fluorescent marker in each experiment were used. For image analysis and quantification, measurements were made using Metamorph Image analysis software ver. 7.0. Average intensity was calculated from integrated intensity and area for each selected area. Quantitation of fluorescence signals from 5 random fields for each treatment was done. An example of an original image used for quantitation is included in the supplementary materials (Supplementary Fig. S2).

**Immunoprecipitation**

HEK 293 cells were lysed with RIPA buffer and centrifuged at 14,000 × g for 15 minutes. The supernatant was recovered and precleared by adding 1 μg of normal IgG premixed with 20 μL of A/G protein bead slurry. The mixture was incubated for 30 minutes at 4 °C and then centrifuged at 1,000 × g for 5 minutes. The supernatant was recovered and mixed with 10 μL of primary antibody and incubated for 1 hour at 4°C. Twenty microliters of A/G bead slurry was then added and incubated at 4°C for 1 hour. Samples were then centrifuged, and supernatants were discarded. The precipitate was boiled for 3 minutes after adding 20 μL of SDS-PAGE sample buffer to release the complex from the beads. Western blotting was then done as described above.

**Homology modeling**

AGBL2 structure was predicted with human carboxypeptidase A1 (CPA1; PDB code: 1V77) as a template. The sequence identity of AGBL2 and CPA1 is 27%. The missing loops were built using the "loop model" building option in the Modeler9v7. The model was refined further by molecular dynamics (MD) simulations, followed by energy minimization using SANDER module of AMBER 10.0. The quality of the refined model was checked with PROCHECK. Docking of the 'EEY' peptide motif was carried out with the SurfFlexDock. MD simulations and energy minimization were done using the AMBER10 package.

**Immunofluorescence**

To assess the levels of detyrosinated tubulin in control versus AGBL2, RARRES1 knockeddown, and/or paclitaxel-treated HEK 293 cells, 50,000 cells were plated on cover slides (Fisher brand microscope cover glass #12-545-100 18CIR-1). After 24 hours, cells were treated with paclitaxel or dimethyl sulfoxide at a final concentration of 5 μmol/L for 2 hours. HEK 293 cells were then fixed in 3.7% paraformaldehyde/PBS for 10 minutes at room temperature, followed by washing the cells 3 times with PBS. Postpermeabilization was done by adding PBST (PBS/Tween 20) and incubating the cells for 5 minutes at room temperature. Cells were then washed 3 times with PBS, followed by the addition of the primary antibody that consisted of a rabbit polyclonal anti–detyrosinated tubulin (ABD Serum) with a final dilution of 1:250. Cells were then washed 3 times with PBS before adding the secondary antibody that consisted of an Alexa 488–conjugated anti-rabbit IgG diluted 1:300 (Invitrogen). 4',6-Diamidino-2-phenylindole (DAPI) was also added at a 1:50 dilution for nuclei detection. Cells were washed 3 times with PBS, and images were obtained using a 60× oil lens on the Olympus FV 300 confocal microscope. Consistent laser intensity or camera exposure levels for each fluorescent marker in each experiment were used. For image analysis and quantification, measurements were made using Metamorph Image analysis software ver. 7.0. Average intensity was calculated from integrated intensity and area for each selected area. Quantitation of fluorescence signals from 5 random fields for each treatment was done. An example of an original image used for quantitation is included in the supplementary materials (Supplementary Fig. S2).

**Results**

**RARRES1 organization**

*RARRES1* is related to the putative carboxypeptidase inhibitor *LAX*, and both genes are adjacent to one another on...
chromosome 3, suggesting that they arose as a result of gene duplication (ref. 14; Fig. 1A). Similar molecules exist in all vertebrates examined; in zebrafish and several other more ancient vertebrates, only one orthologue exists, and this is more closely related to \textit{RARRES1} than to \textit{LXN} (Fig. 1B). These data indicate that \textit{RARRES1} is the ancestral gene and in zebrafish, may, at least, fulfill the function of both \textit{LXN} and \textit{RARRES1}. We found that \textit{RARRES1} exists as 2 mRNA splice-variants: a low abundance variant and an abundant form which encodes for an additional 66 amino acids at the C-terminus (Fig. 1C–E).

\textit{RARRES1} mRNA is decreased in prostate cancer and further in metastatic prostate cancer compared with normal prostate tissue and is induced after neoadjuvant therapy (2). RT-PCR analysis shows that \textit{RARRES1} is expressed in normal prostate and breast cell lines and more differentiated breast cancer cell lines and is very low in aggressive prostate and breast cancer cells with a mesenchymal phenotype (Fig. 2A; Supplementary Table S1). HEK 293 cells express low but still detectable levels of \textit{RARRES1}. In PWRE-1E cells, \textit{RARRES1} is further induced both by retinoic acid (RA) or vitamin D$_3$ (D$_3$) in PWRE-1E cells but not in PC-3 or other more aggressive prostate cancer cells.

Identification of the \textit{RARRES1} interactome

We utilized a tandem affinity purification (TAP) LC/MS-MS approach to characterize the \textit{RARRES1} interactome in HEK 293 cells. Nano-LC-Q-TOF/TOF mass spectrometry and a ProteinPilot software search revealed 9 proteins (including \textit{RARRES1}) identified with a CI of more than 95% (Table 1). Proteins that were also present in a complex isolated from cells stably expressing the empty vector control were removed from the analyses. Several proteins in the \textit{RARRES1} complex regulate microtubule function. These are as follows: the mitotic spindle-associated kinesin eg5/KIF11, an emerging target for cancer therapy; EB1, a microtubule plus end-binding
protein that is regulated by RARRES1 (15); a novel human cytosolic carboxypeptidase member of the ABGL/CCP family (AGBL2), and α-tubulin. The presence of AGBL2, Eg5, and EB1 in the exogenously and endogenously expressed RARRES1 complex was confirmed by Western blot after TAP, RARRES1 immunoprecipitation, and reverse AGBL2 immunoprecipitation (Fig. 2C). Two other interactors, ANKRD26-like family member 1A and Crk-like protein kinase, are likely involved in

Table 1. RARRES1 interactome

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NOTE: List of proteins identified at more than 95% confidence as being present in the RARRES1-TAP produced complex. Proteins that were also present in a complex isolated from cells stably expressing the empty vector control were removed from this list.
the regulation of the membrane-associated actin cytoskeleton (16).

**RARRES1 is a type III membrane protein**

An affinity purified polyclonal antibody raised to residues 43 to 294 of human RARRES1 protein recognizes an approximately 38-kDa protein from PWR-1E whole cell lysate which is present at much reduced levels in PC-3 cells, consistent with mRNA data. The antibody recognizes TAP-tagged RARRES1, DN-RARRES1, and RARRES1-EYFP at their respective predicted molecular masses but does not detect the variant isoform of RARRES1-EYFP. Cell fractionation analysis reveals that approximately 38-kDa RARRES1 is membrane bound; a second, higher molecular weight pool (~50 kDa) is present in the nuclear/cell debris pool, indicating a form which is highly modified and/or insoluble in nonionic detergents (Fig. 2B). Although RARRES1 has a transmembrane domain, its directionality once inserted into the membrane is unclear (www.expasy.ch). We addressed RARRES1 orientation by examining its N-glycosylation status via incubation with PNGase F glycosidase. Unlike E-cadherin, RARRES1 does not undergo a shift in molecular weight when exposed to PNGase F (Fig. 2B), suggesting that, even though it possesses several putative sites for glycosylation, it is not N-glycosylated and that the C-terminal likely faces the cytoplasm and not lumen of the membrane compartment (a type III transmembrane protein). This is consistent with its association with several cytoplasmic proteins (Table 1).

**Regulation of α-tubulin tyrosination**

Other than its presence in GenBank, nothing is known of human AGBL2, though a likely family member, mouse Nna-1, is important in cerebellar degenerative disorders (17). Nna-1 knockout mice have high levels of tyrosinated tubulin in the...
degenerative cerebellum, and it was suggested that Nna-1 might have the characteristics of a tubulin carboxypeptidase (18). However, it is now thought that Nna-1 is involved in the degradation of proteosomally generated peptides (19, 20).

Although significant levels of AGBL2 and other family members exist in HEK 293, PC-3, and PWR-1E cells, Nna-1 (AGTPBP) was not detectable by quantitative PCR (Fig. 2D) and neither itself nor the other AGBL family members were found in the RARRES1 complex (Table 1).

To gain some insight into its potential carboxypeptidase activity, we predicted AGBL2 structure using human CPA1 (PDB code: 1V77) as template. Although the overall sequence identity is only 27% (active site 21%), the predicted tertiary structure of AGBL2 is remarkably similar to CPA1 (Fig. 3A). Importantly, the residues predicted to interact with the zinc coordination atom and the folding in this catalytic site is almost identical. Although the structure of the catalytic site is very similar, the substrate binding cavity of AGBL2 is lengthy and narrow whereas it is bulky and wide for CPA1 (Fig. 3B and C). These structural predictions strongly indicate that AGBL2 is likely to have carboxypeptidase activity. Importantly, extensive molecular simulations indicate that the putative C-terminal \( \alpha \)-tubulin peptide EEY substrate can be docked precisely into the predicted AGBL2 binding pocket (Fig. 3D).

To further test whether AGBL2 has the characteristics of a tubulin carboxypeptidase, we carried out Western blots using antibodies specific for the tyrosinated and detyrosinated forms of \( \alpha \)-tubulin on proteins extracted from HEK 293 control cells and cells in which AGBL2 was knocked down. Several predicted AGBL2 variants and/or possibly degradation fragments are detected in Figure 4A. AGBL2 siRNA resulted in marked knockdown of all AGBL2 variants and fragments. B, RARRRES1 immunoblot depicting endogenous RARRRES1 (35 kDa), using a mouse monoclonal antibody. C and D, immunoblot for detyrosinated \( \alpha \)-tubulin in HEK 293 cells in which RARRRES1 or AGBL2 was knocked down or exogenously expressed. Control Myc, control pGle, Myc-AGBL2, and RARRRES1-pGle transfections were done in duplicate. Loading control was done using a monoclonal anti-\( \alpha \)-tubulin antibody.

Figure 4. RARRRES1 and AGBL2 regulate \( \alpha \)-tubulin tyrosination in HEK 293 cells. A, AGBL2 immunoblot depicting the full-length protein (104 kDa) and other predicted variants (or degradation fragments) at 72, 64, 38, 33, and 31 kDa. Myc-AGBL2 transfection results in a low amount of full-length exogenous protein expression. AGBL2 knockdown results in the disappearance of all AGBL2 variants and fragments. B, RARRRES1 immunoblot depicting exogenous pGle-RARRRES1 (45 kDa) and endogenous RARRRES1 (35 kDa), using a mouse monoclonal antibody. C and D, immunoblot for detyrosinated \( \alpha \)-tubulin in HEK 293 cells in which RARRRES1 or AGBL2 was knocked down or exogenously expressed. Control Myc, control pGle, Myc-AGBL2, and RARRRES1-pGle transfections were done in duplicate. Loading control was done using a monoclonal anti-\( \alpha \)-tubulin antibody.
Figure 5. RARRES1 and AGBL2 regulate the detyrosination cycle of α-tubulin in HEK 293 cells. A, immunofluorescence signal of Alexa 488 probing the detyrosinated α-tubulin (green) and DAPI probing the nucleus (blue) in HEK 293 controls, RARRES1 knockdown, AGBL2 knockdown, RARRES1 overexpression, and AGBL2 overexpression with and without taxol treatment. The color balance and the contrast of all the images combined were optimized using Photoshop CS3 to allow for visualization of both DAPI and detyrosinated tubulin staining on an 8-bit resolution monitor. An example of the original image used for quantitation is included in the supplementary materials (Supplementary Fig. S1); B, average signal intensities of detyrosinated α-tubulin in HEK 293 cells following knockdown or exogenous expression of RARRES1 with and without taxol treatment (*, P < 0.05 and #, P < 0.001); C, average signal intensities of detyrosinated α-tubulin in HEK 293 cells, following knockdown or exogenous expression of AGBL2 with and without taxol treatment (*, P < 0.05; **, P < 0.01; #, P < 0.001; ###, P < 0.0001).
AGBL2 and RARRES1 are α-tubulin tyrosine carboxypeptidase and carboxypeptidase inhibitor, respectively.

**Discussion**

Previous studies indicated a role for RARRES1 in some aspect of cellular proliferation and tumorigenicity (3). However, these groups utilized the variant version of RARRES1 in their studies (the only form identified at the time), and we have shown that the variant mRNA species is present in very small quantities and cannot verify that this transcript is translated into a functional protein. Consequently, it is likely that the major function of RARRES1 is carried out by the full-length isoform.

The RARRES1 parologue LXN, was initially described as a carboxypeptidase inhibitor in the rodent brain and regulates hematopoietic stem cell numbers and lifespan in mice (4, 22). RARRES1 also regulates proliferation and differentiation in adipose-derived mesenchymal stem cells and proliferation and motility in HK1 cells, suggesting a role for this family of genes in the differentiation of stem cells from several different tissues (23). Recombinant LXN can inhibit the activity of the lysosomal carboxypeptidase A (CPA) family members in vitro and can be cocrystallized with them; however, structural analyses and localization patterns of LXN clearly suggest that it is a cytoplasmic protein and thus it is not likely to interact with members of the lysosomal CPA family (24). Because RARRES1 is a type III transmembrane protein, the cytoplasmic localization of its putative carboxypeptidase inhibitor domain would preclude it too from interacting with the lysosomal CPAs. This suggests that its cognate carboxypeptidase(s) is likely active within the cytoplasm. Although little is known about cytoplasmic carboxypeptidases or their substrates, removal of the C-terminal tyrosine of α-tubulin by an unknown carboxypeptidase (the tyrosination cycle) is important in several aspects of microtubule function including kinesin interactions, spindle dynamics, mitosis, and neuronal specification (25). Microtubules containing large amounts of detyrosinated α-tubulin are more stable and resistant to depolymerization by nocodazole and other destabilizing agents (26). Detyrosinated α-tubulin is elevated in aggressive breast and prostate cancers which are often resistant to microtubule-targeted chemointerventions (27). Elevated levels of detyrosinated α-tubulin and A2-tubulin, a highly stable version generated from the precursor detyrosinated form, predominate in normal brain tissue but are also associated with tumors in other tissues, further suggesting a role for the tubulin tyrosination cycle in tumorigenesis (28). Recently, a role for detyrosinated tubulin in epithelial to mesenchymal transitions, important in development, stem cell differentiation, and tumor invasion has been proposed (29). Remarkably, although the tubulin tyrosination cycle has been known to occur for decades, the identity and regulation of tubulin carboxypeptidase have remained a mystery (30, 31).

Our demonstration that AGBL2 is a RARRES1-interacting protein that regulates the tubulin tyrosination cycle implicates both molecules in the regulation of this evolutionarily ancient process and identifies it as a novel target for intervention (Fig. 6).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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