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RanBP10 acts as a novel coactivator for the androgen receptor

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Abstract

Androgen receptor (AR) acts as a ligand-activated transcription factor that regulates the expression of genes involved in prostate development and tumorigenesis. RanBP10 shares significant amino acid sequence similarity with RanBPM that is a well-known AR coactivator. Here, we demonstrate that RanBP10 enhances the ligand-dependent transcriptional activity of AR and forms a complex with AR. RanBP10 together with RanBPM exerted an additive effect on AR transactivation. Overexpression of RanBP10 enhanced transcriptional activity of glucocorticoid receptor, but not estrogen receptor α. RanBP10 was highly expressed in AR-positive prostate cancer LNCaP cells, while RanBPM was abundant in WI-38 and MCF-7 cells rather than prostate cancer cells. RanBP10 was mostly co-localized with RanBPM throughout the cytoplasm and nucleus and formed a protein complex with itself or RanBPM. These results suggest that RanBP10 enhances AR transactivation as a homo-oligomer or a hetero-oligomer with RanBPM.
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Keywords: RanBP10; RanBPM; Coactivator; Androgen receptor; Prostate; Glucocorticoid receptor; Steroid hormone; Nuclear receptor

Testosterone is the predominant circulating androgen and is converted to 5α-dihydrotestosterone (DHT) by 5α-reductase in the prostate [1]. DHT exerts its function through the androgen receptor (AR) that is localized in the cytoplasm as a complex with heat shock proteins. The AR is a member of the steroid hormone receptor superfamily, which includes glucocorticoid receptor (GR) and estrogen receptor α (ERα). Members of this family are composed of three functional domains, an N-terminal domain (NTD), a DNA-binding domain (DBD), and a ligand-binding domain (LBD) [2]. When DHT binds to the LBD of AR, the ligand-bound AR is translocated into the nucleus, binds to an androgen response element in the promoter region of genes involved in maturation of the prostate gland and initiation and progression of prostatic carcinoma, and regulates the expression of these genes as a transcriptional factor. The efficient transcriptional activation of AR is achieved by the recruitment of coactivators. Because AR is expressed in a highly restricted tissue-specific manner, the existence and expression level of coactivator in AR-positive tissues affect the degree of AR transactivation.

RanBPM/RanBP9, which has been isolated as a binding partner of Ran, a small Ras-like GTPase, exerts multiple functions through interaction with various proteins [3–8]. Ran is involved in the nucleocytoplasmic transport [9]. Meanwhile, Ran is identical to ARA24 that enhances the transcriptional activity of AR as a coactivator [10]. Interestingly, RanBPM also acts as an AR coactivator [5]. RanBPM possesses the structural characteristics in order from the N-terminus: the N-terminal proline- and glutamine-rich regions, the SPRY domain, and the LisH-CTLH and CRA motifs [4,11]. On the other hand, a previously uncharacterized cDNA clone encoding a protein with high amino acid sequence similarity to RanBPM has been found in the GenBank database and named RanBP10 after its ability to bind to Ran [7]. Although RanBP10 possesses some structural characteristics of RanBPM, such as a SPRY domain and LisH-CTLH and CRA motifs, the biological significance of RanBP10 remains unclear. In the present study, we report that RanBP10 enhances the transcriptional

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activity of AR in a ligand-dependent manner and exhibits a protein expression pattern different from RanBPM in various cell lines. Furthermore, we determined the subcellular distribution and intracellular localization of RanBP10 and analyzed whether RanBP10 forms a protein complex with itself or RanBPM.

Materials and methods

Cell culture. COS-7 (African green monkey kidney), PC-3 (human AR-negative prostate cancer), and LNCaP (human AR-positive prostate cancer) cells were cultured as described previously [12]. Human cell lines (WI-38, lung fibroblast; HepG2, hepatoma; HEK293, embryonic kidney; and MCF-7, breast cancer) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO_2/95% air atmosphere at 100% humidity unless otherwise indicated.

Construction of vectors. pBluescript SK^+-RanBP10 (KIAA1464) (GenBank AB040897) was kindly provided by Kazusa DNA Research Institute (Chiba, Japan). The N-terminal HA-, Myc-, and FLAG-tagged RanBP10 expression vectors (pcDNA3.1-HA-RanBP10, pcDNA3.1-Myc-RanBP10, and p3xFLAG-RanBP10, respectively) were constructed. Human RanBPM cDNA (GenBank AB055311) was amplified by PCR, and the N-terminal HA- and FLAG-tagged RanBPM expression vectors (pcDNA3.1-HA-RanBPM and p3xFLAG-RanBPM, respectively) were constructed. The pMMTV-LTR-Luc reporter plasmid was constructed by introducing the MMTV-LTR fragment derived from pMAM-neo (Clontech Laboratories, Palo Alto, CA, USA) into pGL4.20 vector (Promega Corp., Madison, WI, USA).

Preparation of anti-RanBP10 and anti-RanBPM antibodies. His-tagged recombinant proteins corresponding to amino acids 367–496 of RanBP10 and amino acids 410–729 of RanBPM were expressed in *Escherichia coli*, and affinity-purified as described previously [13]. Anti-RanBP10 and anti-RanBPM antibodies were raised in mouse and rabbit, respectively.

Reporter assay and Western blot analysis. Luciferase reporter assay was performed using human AR, GR, or ERα expression vector (pcDNA3.1-AR, pcDNA3.1-GR, or pCAGGS-ERα) and luciferase reporter vector (pARE2-TATA-Luc or p3xERE-TATA-Luc) as described previously [12], except for pMMTV-LTR-Luc reporter vector. For detection of exogenous FLAG-RanBP10 or FLAG-RanBPM in reporter assay, cell lysates in passive lysis buffer (Promega Corp.) were subjected to SDS–PAGE, followed by Western blotting with anti-FLAG antibodies (1/5000, M2, Sigma, St. Louis, MO, USA). For detection of endogenous RanBP10, RanBPM, AR, and α-tubulin, various cells were lysed in 20 mM Hepes-NaOH, pH 7.5, containing 150 mM NaCl, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin. Cell lysates were analyzed by Western blotting with anti-RanBP10 (1/1000), anti-RanBPM (1/500), anti-AR (1/3000, N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-α-tubulin (1/5000, DM1A, Sigma) antibodies.

Immunoprecipitation analysis. COS-7 cells (100-mm dish) were transfected with pcDNA3.1-AR (4 μg) or pcDNA3.1-Myc-His (mock vector, 4 μg) and p3xFLAG-RanBP10 (20 μg) for detection of an AR–RanBP10 complex, or pcDNA3.1-HA-RanBP10 (12 μg) or pcDNA3.1-HA-RanBPM (12 μg) and p3xFLAG-RanBP10 (12 μg) for detection of a RanBP10–RanBP10 or RanBP10–RanBPM complex using Metafectene (Biontex Laboratories GmbH, Martinsried/Planegg, Germany) for 5 h. Preparation of cell lysates and immunoprecipitation were performed as described previously [12]. Proteins (500 μg) in the supernatant were incubated with 2 μg of control mouse IgG or anti-AR IgG (AR441, Santa Cruz Biotechnology), or control rat IgG or anti-HA IgG (3F10, Roche Diagnostics, Penzberg, Germany), followed by addition of protein G-Sepharose (GE Healthcare, UK Ltd., Buckinghamshire, England). Proteins bound to the resin were analyzed by Western blotting with anti-AR (1/3000, N-20), anti-FLAG (1/5000), or anti-HA (1/1000) antibodies.

Subcellular fractionation and immunofluorescent microscopy. COS-7 cells were electroporated with p3xFLAG-RanBP10 (12 μg) or pcDNA3.1-Myc-RanBP10 (12 μg) and pcDNA3.1-HA-RanBPM (12 μg) using GenePulser at 950 μF and 220 V (Bio-Rad Laboratories, Hercules, CA, USA). Subcellular fractionation was performed as described previously [12]. Proteins in each fraction were analyzed by Western blotting with anti-HA (1/1000), anti-FLAG (1/5000), anti-triosephosphate isomerase (TPI, 1/1000) [14], or anti-lamin B1 antibodies (1/1000, L-5, Zymed, San Francisco, CA, USA). Immunofluorescent microscopy was performed as described previously [15]. Cells were fixed, permeated, and incubated with mouse anti-Myc IgG (1/1000, 9E10, Santa Cruz Biotechnology) and rat anti-HA IgG (1/1000), followed by immunoreaction with Alexa Flour 488- and 594-conjugated secondary anti-mouse and anti-rat antibodies (1/5000, Molecular probe, Eugene, OR, USA), respectively. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (1 μg/ml), followed by inspection using a fluorescent microscope (Nikon, Tokyo, Japan).

Statistical analysis. The effects of FLAG-RanBP10 and FLAG-RanBPM on AR transactivation were evaluated by one-way ANOVA, and a post hoc analysis was performed by Turkey's test. Analysis was performed with GB-Stat 5.4 software (Dynamic Microsystems, Silver Spring, MD, USA). Data are shown as means ± SD, and differences are considered significant when $p < 0.05$.

Results

Enhancement of the transcriptional activities of AR and GR by RanBP10

RanBPM was previously shown to enhance the transcriptional activity of AR in a ligand-dependent manner [5]. AR transactivation increased with increasing RanBP10 expression level in the presence of ligand in PC-3 cells (three black bars on left in Fig. 1A). When RanBP10 and RanBPM were moderately overexpressed (second and fourth black bars in Fig. 1A, respectively), or highly overexpressed (third and fifth black bars), they enhanced AR transactivation to similar levels. Similar results were obtained when pMMTV-LTR-Luc and COS-7 cells were used instead of pARE2-TATA-Luc and PC-3 cells, respectively (data not shown). Simultaneous overexpression of RanBP10 and RanBPM had an additive effect on AR transactivation. Subsequently, we assessed the effects of RanBP10 on the transcriptional activities of other steroid hormone receptors. RanBP10 enhanced GR transactivation in the presence of dexamethasone (DEX) as well as RanBPM (Fig. 1B, left panel). In contrast, neither RanBP10 nor RanBPM affected 17β-estradiol (E2)-induced ERα transactivation (Fig. 1B, right panel). These results indicate that the functional properties of RanBP10 with respect to AR and GR transactivation are comparable to those of RanBPM.

A protein complex of RanBP10 with AR

To examine whether RanBP10 forms a protein complex with AR, FLAG-RanBP10, and AR were overexpressed in the presence of DHT in COS-7 cells, and immunoprecipitation analysis was performed. Cell lysates

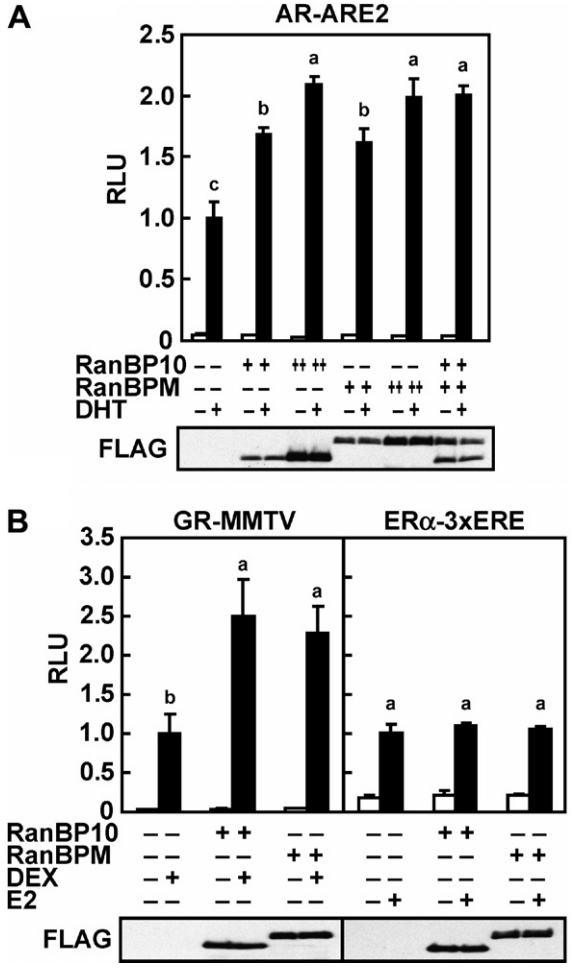

Fig. 1. Effects of RanBP10 on transcriptional activities of steroid hormone receptors. (A) PC-3 cells in steroid-free RPMI1640 medium on 12-well plates were transiently transfected with pcDNA3.1-AR (0.5 μg), pARE2-TATA-Luc (0.5 μg), pRL-TK (0.05 μg), and p3xFLAG-RanBP10 and/or p3xFLAG-RanBPM (1.25 μg or 2.5 μg) for 24 h. (B) (Left panel) PC-3 cells were transfected with pcDNA3.1-GR (0.5 μg), pMMTV-LTR-Luc (0.1 μg), pRL-TK (0.05 μg), and p3xFLAG-RanBP10 (2.5 μg) or p3xFLAG-RanBPM (2.5 μg) for 24 h. (Right panel) PC-3 cells were transfected with pCAGGS-ERα (0.5 μg), p3xERE-TATA-Luc (0.5 μg), pRL-TK (0.05 μg), and p3xFLAG-RanBP10 (2.5 μg) or p3xFLAG-RanBPM (2.5 μg) for 24 h. Cells were incubated in the presence of 10 nM DHT (for AR), 10 nM DEX (for GR), or 10 nM E2 (for ERα) for an additional 24 h, and luciferase activities were determined. Samples used in the luciferase assay were subjected to Western blotting with anti-FLAG antibodies. In the luciferase reporter assay, the amount of DNA was kept constant by addition of p3xFLAG-CMV-26 empty vector. Data are expressed as means ± SD of experiments performed in triplicate. Statistically significant differences ($p < 0.05$) of RLU between the RanBP10 and/or RanBPM plasmid-transfected cells and mock plasmid-transfected cells are indicated by different letters.

were incubated with monoclonal anti-AR IgG (AR441) or unimmunized control mouse IgG, and proteins bound to each IgG were analyzed by Western blotting. Anti-AR IgG specifically co-immunoprecipitated FLAG-RanBP10 together with AR (Fig. 2). Similar results were obtained when polyclonal anti-AR IgG (N-20) was used instead of monoclonal anti-AR IgG (AR441) (data not shown).

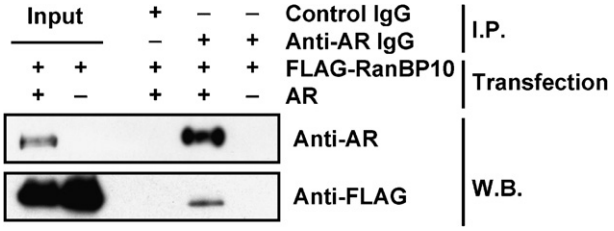

Fig. 2. RanBP10 and AR complex *in vivo*. COS-7 cells were transiently transfected with p3xFLAG-RanBP10 and pcDNA3.1-AR for 5 h, followed by incubation for 28 h. Cells were further incubated for 15 h in the presence of 10 nM DHT. Cell lysates (500 μg) were incubated with monoclonal anti-AR IgG or control mouse IgG, followed by addition of protein G-Sepharose resin. Proteins bound to the resin were analyzed by Western blotting with anti-AR or anti-FLAG antibodies. Each graph is representative of three independent experiments.

These results indicate that RanBP10 forms a protein complex with AR.

Cell type distribution and subcellular localization of RanBP10

RanBP10 mRNA is widely expressed in human tissues and enriched in skeletal muscle [7]. We examined the protein expression pattern of RanBP10 among various cell lines. As shown in Fig. 3A, RanBP10 was highly expressed in AR-positive prostate cancer LNCaP cells compared with HepG2, HEK293, and MCF-7 cells. AR was detected in HEK293 and MCF-7 cells in addition to LNCaP cells. A faint band for RanBP10 was observed in AR-negative prostate cancer PC-3 cells. On the other hand, RanBPM was highly expressed in WI-38 and MCF-7 cells rather than prostate cancer cells. α-Tubulin was detected as an internal control among various cell types. These results indicate that RanBP10 exhibits an expression pattern distinct from that of RanBPM in various cell lines and is preferentially expressed in AR-expressing cells.

RanBPM is localized in the nucleus and cytoplasm [3,4]. To compare the intracellular localization of RanBP10 and RanBPM, subcellular fractionations were performed with COS-7 cells co-expressing FLAG-RanBP10 and HA-RanBPM. FLAG-RanBP10 was predominantly detected in the cytosolic fraction and partly in the nuclear and particulate fractions as well as HA-RanBPM (Fig. 3B). TPI (a cytosolic marker) and lamin B1 (a nuclear marker) were detected only in their corresponding fractions. Furthermore, to assess the intracellular distribution of RanBP10 in intact cells, Myc-RanBP10 and HA-RanBPM were co-expressed in COS-7 cells and doubly stained by anti-Myc and anti-HA antibodies, followed by Alexa Fluor 488- and 594-conjugated secondary antibodies, respectively. Myc-RanBP10 (green) and HA-RanBPM (red) were detected in the DAPI-stained nucleus, and distributed throughout the cytoplasm in a similar punctate pattern (Fig. 3C). The merged image

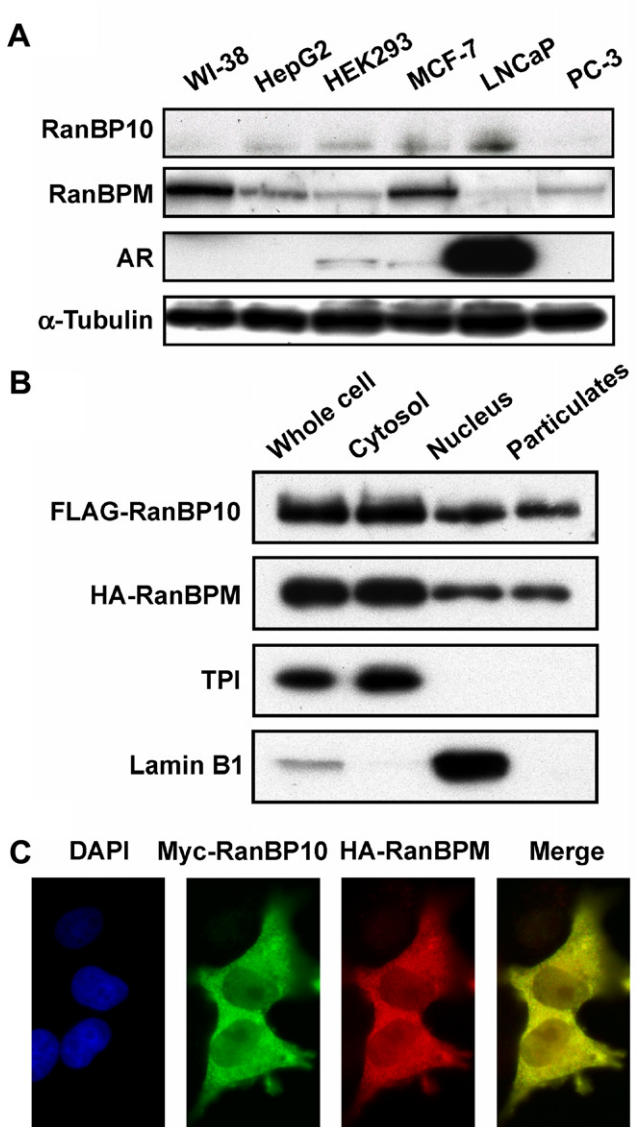

Fig. 3. Cell type distribution and intracellular localization of RanBP10. (A) Cell lysates (20 μg) were analyzed by Western blotting with anti-RanBP10, anti-RanBPM, anti-AR, and anti-α-tubulin antibodies. (B) COS-7 cells were electroporated with FLAG-RanBP10 and HA-RanBPM expression vectors, and incubated in 100-mm dishes for 48 h. Cells were homogenized, and fractionated by differential centrifugation. Proteins (20 μg) in each fraction were analyzed by Western blotting with anti-FLAG, anti-HA, anti-TPI, and anti-lamin B1 antibodies. (C) COS-7 cells were electroporated with pcDNA3.1-Myc-RanBP10 and pcDNA3.1-HA-RanBPM. After incubation for 24 h, cells were fixed, permeated, and incubated with anti-Myc and anti-HA IgGs, followed by incubation with fluorescent-labeled IgGs. The nucleus was stained with DAPI. These results are representative of three independent experiments.

(yellow) showed that Myc-RanBP10 was mostly co-localized with HA-RanBPM.

A protein complex of RanBP10–RanBP10 or RanBP10–RanBPM

Because the LisH-CTLH motif functions as a dimerization interface [16,17], and RanBP10 was mostly co-localized with RanBPM (Fig. 3C), we performed an immunoprecipitation analysis to determine whether RanBP10 forms a homo-oligomer or a hetero-oligomer with RanBPM. Anti-HA antibodies specifically co-immunoprecipitated FLAG-RanBP10 with either HA-RanBP10 or HA-RanBPM, indicating that RanBP10 forms a protein complex with itself or RanBPM *in vivo* (Fig. 4).

Discussion

We have demonstrated that RanBP10 enhances AR transactivation in a ligand-dependent manner and is abundant in AR-positive LNCaP cells. RanBP10 also enhanced the transactivation of GR, but not ERα, in a ligand-dependent manner, consistent with previous report on the biological function of RanBPM [5]. Among members of the steroid receptor superfamily, the DBD and LBD are highly conserved. Coactivators that interact with the DBD or LBD act as common coactivator for multiple steroid hormone receptors. RanBPM binds to not only the NTD but also the DBD of AR [5]. Because the NTD of the receptors is poorly conserved and the DBD of AR exhibits more similarity to that of GR (79%) than ERα (59%) [2], RanBP10 might act as an AR coactivator through binding to the DBD of AR.

The AR is expressed in a limited number of tissues such as prostate, indicating that coactivators specific to AR can act only in AR-positive tissues. RanBP10 was expressed in AR-positive cell lines such as LNCaP and MCF-7 cells, whereas RanBPM was highly expressed in MCF-7 cells, rather than in LNCaP cells. When LNCaP cells are stimulated with synthetic androgen R1881, AR binds to the promoter region of RanBP10 [18]. Interestingly, AR is expressed in a large proportion of primary breast cancers in addition to prostate cancers, and increased androgen signaling represses the growth of some breast cancer cells [19,20]. These results suggest that RanBP10 is amplified through AR in AR-positive cells and that RanBP10 rather than RanBPM contributes to the development and

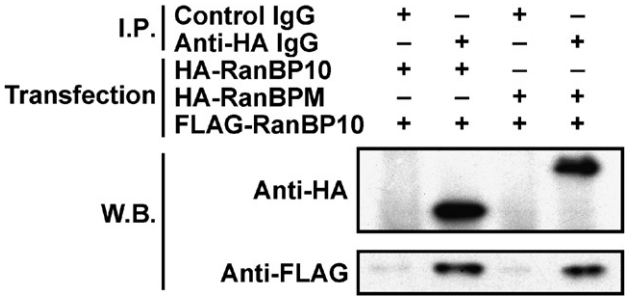

Fig. 4. A protein complex of RanBP10. COS-7 cells were transiently transfected with expression vectors for 5 h, followed by incubation for 43 h. Whole cell lysates were prepared and incubated with monoclonal rat anti-HA IgG or control rat IgG, followed by addition of protein G-Sepharose resin. Proteins bound to the resin were analyzed by Western blotting with anti-HA (upper panel) and anti-FLAG (lower panel) IgGs. These results are representative of three independent experiments.

progression of androgen-dependent prostate cancer cells as an AR coactivator. On the other hand, RanBP10 and RanBPM might contribute to inhibition of breast cancer cell growth through AR-mediated androgen signaling.

Immunoprecipitation analyses indicated that RanBP10 formed a protein complex with itself or RanBPM. Furthermore, we observed an additive effect of RanBP10 and RanBPM on enhancement of AR transactivation. These results suggest that RanBP10 contributes to enhancement of AR transactivation as a protein complex with itself or RanBPM. On the other hand, although RanBPM activates Ras/Erk signaling pathway through interaction with hepatocyte growth factor receptor, MET, RanBP10 fails to induce the signaling despite binding to MET [6,7]. Thus, RanBP10 and RanBPM exhibit a competitive effect on the MET signaling cascade. Taken together, our data indicate that the physiological functions of RanBP10 and RanBPM are linked and thus difficult to examine separately.

Acknowledgments

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