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Detection of recombinant and endogenous mouse melatonin receptors by monoclonal antibodies targeting the C-terminal domain

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ABSTRACT

Melatonin receptors play important roles in the regulation of circadian and seasonal rhythms, sleep, retinal functions, the immune system, depression and type 2 diabetes development. Melatonin receptors are approved drug targets for insomnia, non-24h sleep-wake disorders and major depressive disorders. In mammals, two melatonin receptors (MTRs) exist, MT_1 and MT_2, belonging to the G protein-coupled receptor (GPCR) super-family. Similar to most other GPCRs, reliable antibodies recognizing melatonin receptors prooved to be difficult to obtain. Here we describe the development of the first monoclonal antibodies (mABs) for mouse MT_1 and MT_2. Purified antibodies were extensively characterized for specific reactivity with mouse, rat and human MT_1 and MT_2 by western blot, immunoprecipitation, immunofluorescence and proximity ligation assay. Several mABs were specific for either mouse MT_1 or MT_2. None of the mABs cross-reacted with rat MTRs, and some were able to react with human MTRs. The specificity of the selected mABs was validated by immunofluorescence microscopy in three established locations (retina, suprachiasmatic nuclei, pituitary gland) for MTR expression in mice using MTR KO mice as control. MT_2 expression was not detected instead in mouse insulinoma MIN6 cells or pancreatic beta-cells. Collectively, we report the first monoclonal antibodies recognizing recombinant and native mouse melatonin receptors that will be valuable tools for future studies.

Keywords: melatonin / melatonin receptor / G protein-coupled receptor / monoclonal antibodies

Introduction

Melatonin receptors belong to the G protein-coupled receptor super-family, which preferentially couple to $G\alpha_{i/o}$ proteins [1]. The melatonin receptor (MTR) subfamily is composed of three members in mammals: MT_1 and MT_2, which are both binding to the neurohormone melatonin with high affinity [2], and GPR50, which has 70% sequence homology with MT_1 and MT_2 but lost the capacity to bind melatonin during evolution due to modifications in the second extracellular loop [3,4].

MT_1 and MT_2 are involved in various biological functions including the regulation of biological rhythms, sleep, pain, retinal, neuronal and immune functions [1,5]. Melatonin receptor knockout mice show deficits in many of these functions [6]. Alteration of MTR function or expression in humans is associated with depression [7], Alzheimer's disease [8-10] and type 2 diabetes [11].

To study the function of proteins and to discriminate between closely related subtypes like MT_1 and MT_2, antibodies are indispensable tools. Yet, reliable and specific antibodies recognizing GPCRs are notoriously difficult to obtain. Unfortunately, this is also the case for MT_1 and MT_2. GPR50 escaped from this rule since its long carboxyl terminal domain facilitated the successful generation of highly specific and sensitive antibodies [12], which boosted progress in its study [13,14]. In the case of MT_1 and MT_2, low expression levels typically observed in tissues render their detection even more difficult [15].

Currently available antibodies include the 536-antibody from our lab recognizing specifically the recombinant and endogenously expressed human MT_1 [7,10,16-23] but neither the human MT_2 nor the mouse, rat or hamster MT_1 (personal communication RJ) and two antibodies from the Fraschini lab [7-9,23-26]. These were generated against the human MT_1 and MT_2, and cross-react with rat but not with the mouse receptors. Other, commercially available, antibodies are poorly characterized and key data for them are generally unavailable, such as

their detection of recombinant MTRs or immunoreactivity (IR) with established tissues of melatonin receptor expression in wild type and in MTR knockout mice as a control. Moreover, all validated antibodies directed against MTRs are polyclonal generated in rabbits, and none of them reacts with the mouse MTRs. Hence, more anti-MTRs antibodies are necessary, especially against those of murine origin. Taking into account that the vast majority of studies on melatonin effects are conducted in mice, reliable antibodies against mice MTRs are of urgent need. We therefore decided to develop monoclonal antibodies (mABs) for mouse MT_1 and MT_2 using their C-terminal regions fused to Glutathione-S-transferase (GST) as immunogen.

MATERIAL AND METHODS

Design and production of GST fusion proteins

Glutathione-S-transferase (GST) fusion proteins composed of GST fused at its carboxyl-terminus with the cytoplasmic domains of the mouse MT_1 (amino acid residues 302 to 353) (GST-mMT_1Cter) or MT_2 (amino acids 312 to 364) (GST-mMT_2Cter) were constructed. GST constructs were expressed in *Escherichia coli* and purified on immobilized glutathione according to standard protocols.

Generation of monoclonal antibodies

A 1:1 mixture of GST-mMT_1Cter and GST-mMT_2Cter fusion proteins (25 µg total amount) was used for immunization of 5 Balb/c mice. Standard PEG fusions with splenocytes harvested from the three mice with the strongest immune response, yielded a total of 28 hybridoma cell lines that were specific for either MT_1 or MT_2. Primary screening was done in a multiplex assay format using the Meso Scale Discovery (MSD) electrochemiluminescence platform. Each hybridoma clone was simultaneously assayed on the injected GST-mMT_1Cter and GST-

mMT_2Cter fusion proteins, as well as on free GST and a non-related GST-fusion protein to eliminate GST-reactive clones. Selected clones were subcloned, and antibodies were purified from hybridoma supernatant using HiTrap Protein G columns (GE Healthcare). Five of the 7 clones with a positive signal namely mAB-A06, mAB-A84, mAB-H04, mAB-J50 and mAB-I81, were further characterized.

Anti-tag antibodies, DNA constructs and mouse models

Mouse anti-FLAG M2 was from Sigma-Aldrich (F7425); rabbit anti-Myc from Upstate Labs and from Santa-Cruz (sc-789), or mouse monoclonal from Roche. Fluorescent secondary antibodies Alexa Fluor-555 anti-mouse and Alexa Fluor-647 anti-rabbit were from Life Technologies (A31570 and A31573, respectively). The expression plasmid for human FLAG-hMT_1 and MYC-hMT_2, those for mouse FLAG-mMT_1, MYC-mMT_1 and mMT_2-FLAG and those for rat rMT_1-FLAG and rMT_2-FLAG were described previously [27-29]. Melatonin receptor MT_1 and MT_2 double KO mice were generated in the C57/bl6 background [30]. MT_2 KO mice used for experiments with pancreatic slices were kindly provided by Dr. Gianluca Tosini (Morehouse School of Medicine, Atlanta, GA) [31]. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the experimental protocols were approved by the local institutional research animal committee.

Cell culture and transfection

HEK293T cells were grown in complete medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4.5g/L glucose, 100 U/mL penicillin, 0.1mg/mL streptomycin and 1mM glutamine) (Invitrogen, CA). Transient transfections were performed using JetPEI (Polyplus Transfection, France), according to manufacturer's instructions.

SDS-PAGE and Western Blotting

48 hours post-transfection, HEK293T cells expressing mouse, human or rat MTRs were collected into lysis buffer (62.5 mM Tris/HCl pH 6.8, 5% SDS, 10% glycerol, 0.005% bromophenol blue), as previously described [32]. Brain tissues were removed and immediately processed for preparation of crude membranes. Briefly, tissues were homogenized using a Polytron homogenizer in TEM buffer (75mM Tris pH7.5; 5mM EDTA; 12.5mM $MgCl_2$), followed by ultracetrifugation (51,500 x g, 45 min) and the remaining pellet was ressuspended in TE buffer (75mM Tris pH7.5; 5mM EDTA). Denatured proteins were resolved in 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted using the mABs (2 µg/mL) or the polyclonal rabbit anti-FLAG or anti-MYC antibodies. Immunoreactivity is revealed using secondary antibodies coupled to 680 or 800 nm fluorophores (LI-COR Biosciences, Lincoln, NE, USA), and membranes were read in the Odyssey LI-COR infrared fluorescent scanner (LI-COR Biosciences).

Immunofluorescence microscopy

Tissues from WT and MT_1/MT_2 KO mice were collected in the morning, at Zeitgeber Time (ZT) 3h (ZT is defined relative to the light cycle, with ZT0 representing the time lights are turned on, and ZT12= lights off), as this was time with maximum melatonin binding sites detected in the hypothalamic suprachiasmatic nucleus of mice [33]. Mice were anesthetized (ketamine/xylazine solution; 100mg/kg-10mg/kg) and perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) and the brain and eye balls were collected, post-fixed in 4% PFA (24h, 4°C) and cryoprotected in 30% sucrose solution (24h, 4°C). Tissues were included in freezing OCT medium (Tissue-Teck), snap frozen and kept at -80°C until processed in the cryostate (CM350S, Leica) to obtain 10 µm-thick sections. Immunofluorescence analysis was also performed in HEK293T cells expressing MTRs. 48 hours post-transfection cells were fixed in 2% PFA (15 min, -20°C) and permeabilized with TritonX-100 (0.2%, 1 h), as described [34].

Tissues slices and fixed cells were blocked with horse serum and incubated with mABs (10 µg/mL, overnight, 4°C), followed by incubation with anti-TAG antibodies (anti-FLAG 1:500, Sigma-Aldrich; anti-MYC (1:500, Santa Cruz) or anti-BIP (endoplasmatic reticulum marker; 1:3000, Sigma-Aldrich) antibody and the secondary antibodies Alexa 555-fluorophore-coupled anti-mouse and Alexa 647-fluorophore-coupled anti-rabbit (1:200, 2h, RT). DAPI (1:3000, 5 min; Santa Cruz Biotechnology, Dallas, TX, USA) was used to stain the cell nuclei. Insulin was detected with a guinea pig anti-insulin antibody (1:200; DAKO, Glostrup, Denmark) followed by a goat anti-guinea pig IgG conjugated to Alexa 568 (1:200; Molecular Probes #A11075, Eugene, OR, USA). The slides were mounted and analyzed by fluorescence confocal microscopy (Axion, Carl Zeiss, Jena, Germany; Leica DMI600 spinning disk CSU-X1M1) or by slide scanner (Lamina, Perkin Elmer). Images were analyzed using ImageJ software (National Institutes of Health, USA).

Proximity Ligation Assay (PLA)

Transfected HEK293T cells, plated onto glass coverslips, were fixed with 4% PFA for 15 min (RT). Fixed cells were permeabilized with 0.2% Triton X-100 for 10 min, followed by blocking in PBS containing 3% BSA (1h, RT). Cells were incubated with mABs A06 or A84 (1µg/ml) or mouse anti-FLAG (1:1000, Sigma-Aldrich) or mouse anti-MYC (1 :1000, Santa Cruz) and goat anti-$5HT_{2C}$ (1:100, Santa Cruz) antibodies overnight at 4°C. PLA was conducted using Duolink® In Situ-Fluorescence kit (Sigma-Aldrich), following the instructions of the manufacturer. Images were captured using a confocal microscope and analyzed with ImageJ software (NIH, Bethesda).

Immunoprecipitation

Crude membranes were prepared from HEK293T cells transiently expressing mMT_1 or mMT_2 were labeled with 2-[^{125}I]iodo-melatonin (400 pM) (PerkinElmer, Life Sciences) as described [35]. Labeled receptors were solubilized with 1% digitonin, a detergent known to maintain melatonin receptors in a native conformation, and cleared lysates incubated with purified mAbs (1µg/mL) overnight at 4°C as previously described [8]. Protein A-agarose was added for 2 h at 4°C to precipitate antibody-receptor complexes. Precipitates were washed twice with ice-cold buffer (75 mm Tris pH 7.4, 12 mm MgCl2, 2 mm EDTA, 0.2% digitonin) and then counted using a γ-counter. Immunoprecipitated receptors from cells previously treated or not with melatonin (100 nM, 15min) were also accessed by western blot using anti-TAG antibodies.

[^{35}S]GTPγS binding

[^{35}S]GTPγS binding was determined from crude membranes prepared from CHO cells stably expressing mMT_1 or mMT_2 as previously described [36]. Briefly, the reaction was performed in buffer containing 20mM HEPES (pH 7.4), 100mM NaCl, 3mM MgCl2, 20 mg/ml saponin, 3 mM GDP, 0.3nM [^{35}S]GTPγS, with or without 1 µM melatonin, in the presence or absence of mABs, for 1h at room temperature. The reaction was stopped by addition of 1 ml of ice-cold buffer containing 10mM Tris–HCl (pH 8.0), 100mM NaCl, 20mM $MgCl_2$, 0.1mM GTP. Bound and free radioactivity was separated by filtration over GF/F glass fibre filters (Whatman).

RESULTS AND DISCUSSION

To obtain mABs against mMT_1 and mMT_2, we expressed in *E. coli* carboxyl terminal GST fusion proteins containing the 52 or 53 C-terminal amino acids of mouse MT_1 and MT_2 (GST-mMT_1Cter and GST-mMT_2Cter), respectively, which show 23% sequence homology (Fig. 1A).

Mice were immunized with the purified fusion proteins. Out of 28 hybridoma cell lines obtained, clones A06, A84, H04, J50 and I81 were retained for further evaluation based on their ability to produce mABs recognizing either GST-mMT_1Cter and GST-mMT_2Cter, but not GST alone by using the Meso Scale Discovery (MSD) technology platform (Supplementary Fig. 1). The reactivity of the five selected mABs was then tested by western blot (WB) in whole-cell lysates of HEK 293T cells expressing, or not, epitope-tagged mMT_1 or mMT_2 (Fig. 2, Fig. 3). mAB-A06 and mAB-J50 recognized mMT_1 but did not show any IR towards lysates of non-transfected cells or cells expressing mMT_2 (Fig. 2A,B). Both mABs and the anti-TAG antibody recognized immunoreactive bands with apparent molecular weights of about 50 and 110 kDa, which most likely correspond to the monomeric and dimeric forms of mMT_1. Further bands migrated at high-molecular weights most likely correspond to receptor oligomers. mAB-A06 recognized also hMT_1 with a similar monomer/dimer pattern as the anti-TAG antibody (Fig. 2C). There was no cross-reactivity with rMT_1 (Fig. 2D).

mAB-A84, mAB-H04 and mAB-I81 readily recognized mMT_2 but did not show any IR against lysates of non-transfected cells or cells expressing mMT_1 (Fig. 3A,B). All three mABs and the anti-tag antibody consistently recognized a doublet band in the range of 55 to 65 kDa and another broad band at 100 to 120 kDa corresponding most likely to different monomeric and dimeric receptor species, respectively. Additional, high-molecular weight oligomeric receptor forms were also detected. hMT_2 and rMT_2 were not recognized by any of the three mABs (Fig. 3C,D). Collectively, these are the first antibodies which specifically recognize either recombinant SDS-denatured mMT_1 or mMT_2 by WB. mAB-A06 recognizes also the hMT_1 receptor, which has 73% (38/52 residues) sequence homology with the mMT_1-Cter (Fig. 1B) .

We next performed confocal immunofluorescence (IF) microscopy experiments in fixed and Triton X-100 permeabilized HEK 293T cells expressing, or not, epitope-tagged MT_1 or MT_2 (Fig. 4, Fig. 5). Co-staining of cells with anti-tag antibodies and mAB-A06 or mAB-J50 showed similar labelling in intracellular compartments and at the cell surface expressing mMT_1 but no staining

of non-transfected cells in the same frame (Fig. 4A,B). Both mABs did not immunolabel cells expressing mMT_2 (Fig. 4B). Cells expressing hMT_1 were stained with both mABs, whereas cells expressing rMT_1 were negative in both cases (Fig. 4C,D). The IR to human receptors revealed to be specific, as virtually no IR was detected in hMT_2 expressing cells (Fig. 4E). In cells expressing mMT_2, mAB-A84, mAB-H04 and mAB-I81 all showed positive IR at the cell membrane and intracellular compartments, which was superimposable with the IR detected with the anti-tag antibodies (Fig. 5A). No staining was observed in non-transfected cells in the same frame (Fig. 5A) or in cells expressing mMT_1 (Fig. 5B). Cells expressing hMT_2 were immunostained with mAB-A84 and mAB-I81 (Fig. 5C), while cells expressing rMT_2 or hMT_1 were negative for all three mABs (Fig. 5D,E). Taken together, the mABs recognized specifically either recombinant mMT_1 or mMT_2 by immunofluorescence microscopy at the cellular level with an comparable pattern to anti-tag antibodies. mAB-A06 and mAB-J50 cross-reacted with hMT_1 (Fig. 1B) and mAB-A84 and mAB-I81 with the hMT_2 (Fig. 1C). None of the mABs cross-reacted with the rat receptors. In order to analyze the subcellular distribution of mMTRs, higher magnitude images were taken and analyzed by co-localization with the endoplasmatic reticulum (ER) marker, BIP. As shown in Figure 6, few co-localization of mMTRs with the ER is observed, as the majority of receptors are located at the plasma membrane.

The proximity ligation assay (PLA) is increasingly applied to detect proximity between two proteins at the cellular level in a sensitive manner. To verify the compatibility of the mABs with this technique, we probed the proximity between the serotonin m5-HT_{2C} receptor and mMT_1 or mMT_2, two heterodimers that have been recently described [37]. Positive PLA signals were observed between the mMT_1-specific mAB-A06 and anti-5-HT_{2C} antibodies in HEK293T cells co-expressing mMT_1 and m5-HT_{2C} receptors but not in mock-transfected cells (Fig. 7A, left and middle panel). Anti-FLAG antibodies were used as positive control (Fig. 7A, right panel). Similarly, positive PLA signals were detected between the MT_2-specific mAB-A84 and anti-5-HT_{2C} antibodies in HEK293T cells co-expressing mMT_2 and m5-HT_{2C} receptors but not in mock-transfected cells (Fig. 7B, left and middle panel). Anti-MYC antibodies were here used as

positive control (Fig. 7B, right panel). The number of PLA signals was comparable to those reported for other GPCR heterodimeric complexes [38,39]. The lower staining observed for the MT_1/5-HT_{2C} dimer compared to MT_2/5-HT_{2C} might be explained by the higher $BRET_{50}$ value (lower propensity to form dimers) of the former, as previously reported [37]. Collectively, mAB-A06 and mAB-A84 are able to detect heterodimeric complexes involving mMT_1 or mMT_2, respectively.

To verify whether the mABs are able to immunoprecipitate native mMT_1 and mMT_2 independently expressed in HEK293T cells we first labeled them with the specific radioligand 2-[^{125}I]iodo-melatonin and then solubilized them with digitonin. Lysates were incubated with mABs, immunocomplexes precipitated with protein A-agarose beads and the amount of the radioactively labeled receptor in precipitates determined. mAB-A06 or mAB-J50 precipitated around 8% of the labelled mMT_1 receptor, which is higher than the amount precipitated by the anti-FLAG antibody. mAB-A84, mAB-H04, mAB-I81 and anti-MYC precipitated between 12 and 18% of the labelled mMT_2 receptor (Fig. 8A-B). Neglectable signals were observed with non-relevant control IgGs, confirming the specificity of the assay (Fig. 8A-B, background). These data show that the mABs recognize the native, digitonin-solubilized melatonin receptors. Because this assay was performed in the presence of the ligand, we wondered whether the activation state of the receptor could impact the ability of mABs to recognize the antigen, as they target the C-terminal domain of the receptors which is usually involved in the recruitment of signaling molecules upon activation. Imunoprecipitation assay revealed by WB in activated *vs.* non-activated cells show that there is no impact of receptor activation on the efficiency of the antibodies in recognizing their target (Fig. 8C-D). The other way round was also investigated, *i.e.* whether the antibodies could affect the activation of the receptors. G protein activation accessed by [^{35}S]GTPγS binding assay revealed no difference on melatonin-induced activation of mMT_1 or mMT_2 in the presence of any mABs (Fig. 8E-F).

We next detected melatonin receptor expression in mouse retina (Fig. 9A). The IR detected with the MT_1-specific mAB-A06 and mAB-J50 and with the MT_2-specific mAB-A84, mAB-H04 and mAB-I81 was restricted to the outer segment, to single cells of the outer plexiform layer (OPL) and to the ganglion cell layer (GCL). Only background staining was observed instead in MT_1 and MT_2 double knockout mice (MTR dKO). These results indicate that our mABs detect endogenously expressed receptors in the mouse retina with an expression pattern in agreement with previous reports [31,40]. The hypothalamic suprachiasmatic nuclei (SCN) and the pituitary gland are two additional expression sites of MTRs [41-45]. Immunostaining of both areas with mAB-J50 and mAB-H04 resulted in the labeling of distinct cells in wild type mice, but not in MTR dKO mice, which only displayed some background signals (Fig. 9B,C). Attempts to detect MTRs by WB in lysates of mouse brain regions of reported MTR mRNA expression [1] (cerebellum, hippocampus, hypothalamus, striatum, cortex) were instead inconclusive as several candidate bands with similar intensity were detected in wild-type and MTR dKO mice illustrating the challenge to detect MTRs in lysates of brain regions where these receptors are only expressed in a subset of cells (Supplementary Fig. 2).

Genetic variants of the *MTNR1B* gene encoding MT_2 have been associated with type 2 diabetes risk [11]. Pancreatic beta-cells were proposed to be a main site of MT_2 action involved in the regulation of glucose homestasis [46] but this suggestion remains controversial, in part due to difficulties in demonstrating the expression of the MT_2 protein in these cells [47]. For example, in the rat MIN6 beta-cell line melatonin-induced cAMP and insulin secretion was reported [48,49]. RNA sequencing data on the expression levels of *MTNR1A* and *MTNR1B* genes coding for MT_1 and MT_2 receptors, respectively, in different beta-pancreatic cell lines including MIN6 cells and mouse and rat islets revealed expression levels close to background levels (Supplementary Table I). In MIN6 cell lysates the MT_2-specific mAB-H04, which does not recognize rat MT_2, detected bands similar to those detected in the rat INS1 cell line, which was used as negative control (Supplementary Fig. 3A). Similarly, we were unable to detect MT_2 expression in MIN6 cells by IF (Supplementary Fig. 3B). Furthermore, mAB-H04 did not reveal

any specific IR in pancreatic sections of WT and MT_2 KO mice (Supplementary Fig. 3C). Taken together, our data indicate that mouse pancreatic beta cells do not express amounts of MT_2 that are detectable with our specific anti-MT_2 mAB-H04.

CONCLUSION

The novel mABs against MTRs described here specifically recognize either mMT_1 or mMT_2 without any cross-reactivity between the two receptors, and will open new venues for their study by enabling the assessment of their protein expression levels in different mouse tissues, which is currently poorly defined by other methods [1,50]. The expression profile of melatonin receptors at the protein level has been systematically described only in the rat brain [26] and in humans only for specific peripheral and central sites [7-10,17-23,25]. Due to the expression of receptors in a subset of cells in a given tissue, immuno-fluorescence and –histological methods are expected to be most successful in detecting these receptors in tissues. Further significant advances may result from co-immuno staining of MTRs in human tissues, *e.g.* for detection of MT_1/MT_2, $MT_2/5\text{-}HT_{2C}$ and MT_1/GPR50 heterodimers or their co-localization with other cell-type specific antigens.

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AUTHOR CONTRIBUTIONS

E.C., A.I., M.L., A.F, P.K, F.G., J.L.G., K.K. and R.A. performed research and/or analyzed data; P.D. provided reagents; R.J. and E.C. wrote the manuscript; E.C., A.I., P.D., and M.S. reviewed and/or edited the manuscript and contributed to discussion; M.S., P.D. and R.J. managed the project and contributed to fund raising.

CONFLICT of INTEREST

All the authors declare no conflict of interest.

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Figure legends

Fig. 1. Alignment of the amino acid sequences of the mouse and human melatonin receptors. The carboxyl terminal (Cter) containing the 52 or 53 last amino acids of the mMT_1 and mMT_2 are shown. (**A**) Alignment between mMT_1Cter and mMT_2Cter (23% sequence homology). (**B**) Alignment between mMT_1Cter and hMT_1Cter (73% sequence homology). (**C**) Alignment between mMT_2Cter and hMT_2Cter (54% sequence homology). Amino acids are colored according to their physicochemical properties. Alignments were generated with the software available at the gpcr.db [51].

Fig. 2. Immunoreactivity of mAB-A06 and mAB-J50 in Western Blots with recombinant receptors. Whole-cell lysates of HEK 293T cells expressing, or not, epitope-tagged mMT_1 (**A**), mMT_2 (**B**), hMT_1 (**C**), and rMT_1 (**D**) were used to test the immunoreactivity of mAB-A06 and mAB-J50. Anti-FLAG or anti-MYC antibodies were used to confirm the presence of the corresponding receptor in the lysates.

Fig. 3. Immunoreactivity of mAB-A84, mAB-H04 and mAB-I81 in Western Blots with recombinant receptors. Whole-cell lysates of HEK 293T cells expressing, or not, epitope-tagged mMT_2 (**A**), mMT_1 (**B**), hMT_2 (**C**), and rMT_2 (**D**) were used to test the immunoreactivity of mAB-A84, mAB-H04 and mAB-I81. Anti-FLAG or anti-MYC antibodies were used to confirm the presence of the corresponding receptor in the lysates.

Fig. 4. Immunofluorescence microscopy analysis of mAB-A06 and mAB-J50 with recombinant melatonin receptors. Fixed HEK 293T cells expressing epitope-tagged mMT_1 (**A**), mMT_2 (**B**), hMT_1 (**C**), rMT_1 (**D**) or hMT_2 (**E**) were incubated with mAB-A06 and mAB-J50, followed by incubation with anti-TAG antibodies, and analysed by confocal immunofluorescence microscopy. The cell nuclei are stained with DAPI (blue). Scale bar: 10 μm.

Fig. 5. Immunofluorescence microscopy analysis of mAB-A84, mAB-H04 and mAB-I81 with recombinant melatonin receptors. Fixed HEK 293T cells expressing epitope-tagged mMT_2 (**A**), mMT_1 (**B**), hMT_2 (**C**), rMT_2 (**D**) or hMT_1 (**E**) were incubated with mAB-A84, mAB-H04 and mAB-I81, followed by incubation with anti-TAG antibodies, and analysed by confocal immunofluorescence microscopy. The cell nuclei are stained with DAPI (blue). Scale bar: 10 μm.

Fig. 6. Immunofluorescence microscopy analysis of subcellular localization of recombinant melatonin receptors. Fixed HEK 293T cells expressing mMT_1 (**A**) or mMT_2 (**B**) were incubated with mAB-A06 (**A**) or mAB-H04 (**B**), followed by incubation with anti-BIP antibody (ER marker), and analysed by confocal immunofluorescence microscopy. The cell nuclei are stained with DAPI (blue). Open white arrowheads indicate absence of co-localization; closed white arrowheads indicate co-localization. Scale bar: 10 μm.

Fig. 7. Proximity ligation assay with mABs against mMT_1 and mMT_2 to detect heterodimeric complexes with 5-HT_{2C} receptors. Fixed HEK 293T cells co-expressing m5-HT_{2C} and FLAG-tagged mMT_1 (**A**) or MYC-tagged mMT_2 (**B**) were submitted to *in situ* PLA using mAB-A06 (**A**) or mAB-A84 (**B**) together with anti-m5-HT_{2C} antibodies and analysed by confocal fluorescence microscopy. Mock transfected cell were used as negative controls and anti-TAG antibodies as positive controls. The cell nuclei are stained with DAPI (blue). Scale bar: 10 μm.

Fig. 8. Immunoprecipitation and function of mMT_1 and mMT_2 in the presence of mABs. HEK 293T cells expressing epitope-tagged mMT_1 (**A**) or mMT_2 (**B**) were labeled using [^{125}I]-melatonin and submitted to immunoprecipitation using mAB-A06, mAB-J50 or anti-FLAG (**A**), mAB-A84, mAB-H04, mAB-I81 or anti-MYC (1µg/mL each) (**B**). Background was defined by using non-relevant control IgGs. Data are expressed as percentage of receptors in the input lysates. Immunoprecipitated mMT_1 (**C**) or mMT_2 (**D**) receptors from cells in the presence or absence of the ligand were also accessed by western blot. No consistent difference in the amount of immunprecipiated receptors was found when analysing 3 independent experimental replicates. mABs have no impact on the function of mMT_1 (**E**) or mMT_2 (**F**) accessed by [^{35}S]GTPγS assay. Data are expressed as mean ± S.E.M of 3 independent experiments.

Fig. 9. Immunofluorescence microscopy analysis of endogenously expressed melatonin receptors. Immunoreactivity of mAB-A06, mAB-J50, mAB-A84, mAB-H04 and mAB-I81 in the retina (**A**), SCN (**B**) and pituitary gland (**C**) from WT or MTR dKO mice. White arrowheads indicate immunoreactive cells. The cell nuclei are stained with DAPI (blue). Scale bar: 100 µm. OS = outer segment; ONL = outer nuclear layer; OPL = outer plexiform layer; INL = inner nuclear layer; GCL = ganglionar cell layer; 3V = third ventricule.

Figure 1

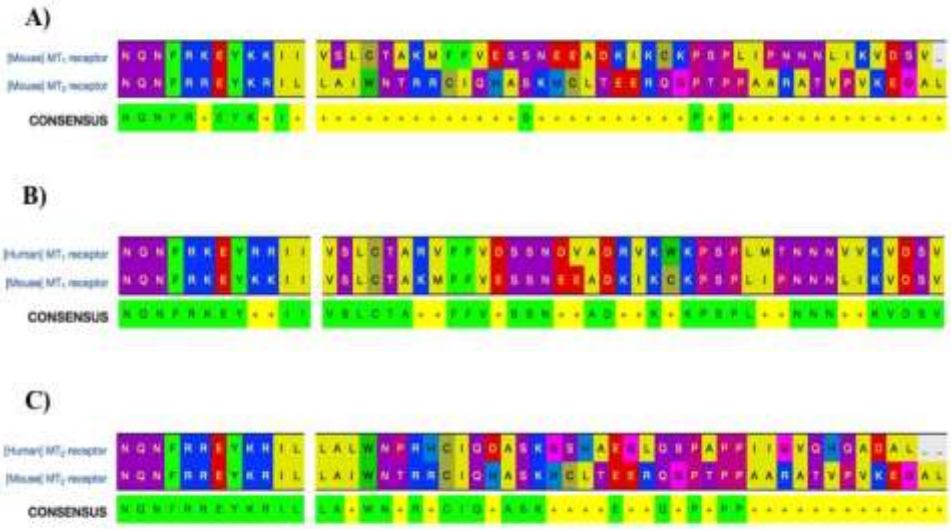

Figure 2

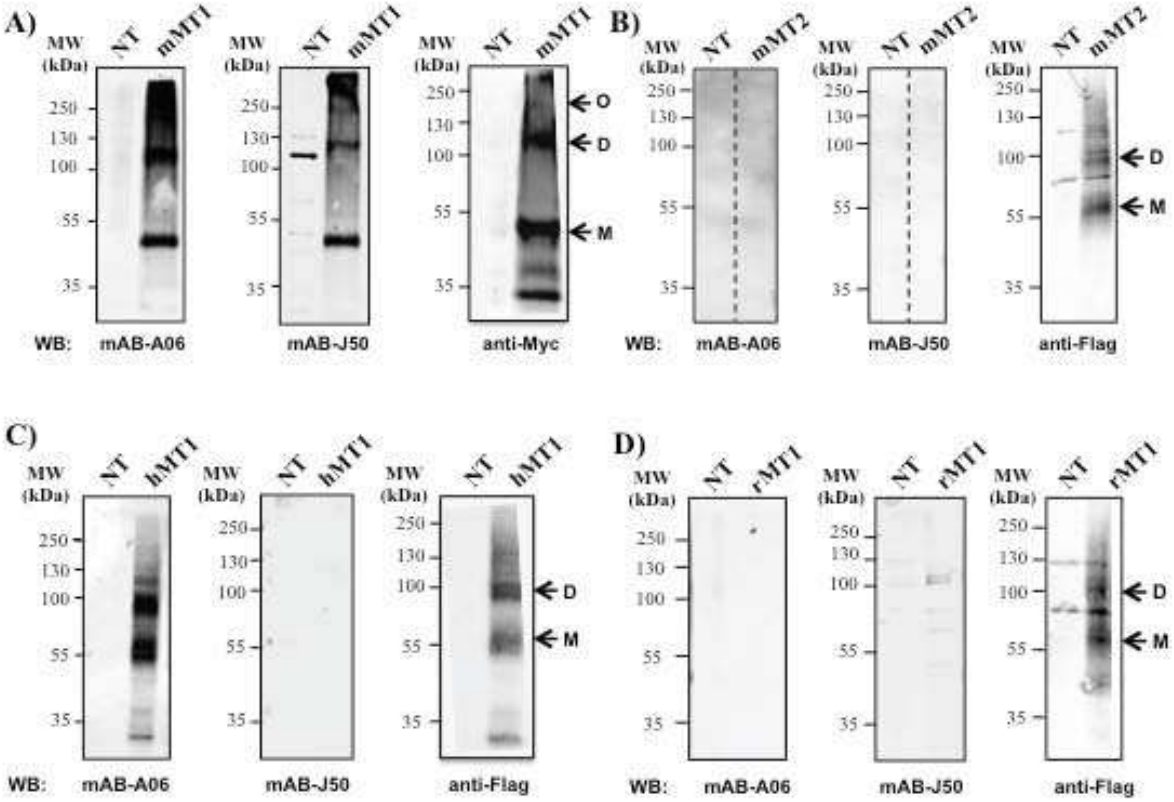

Figure 3

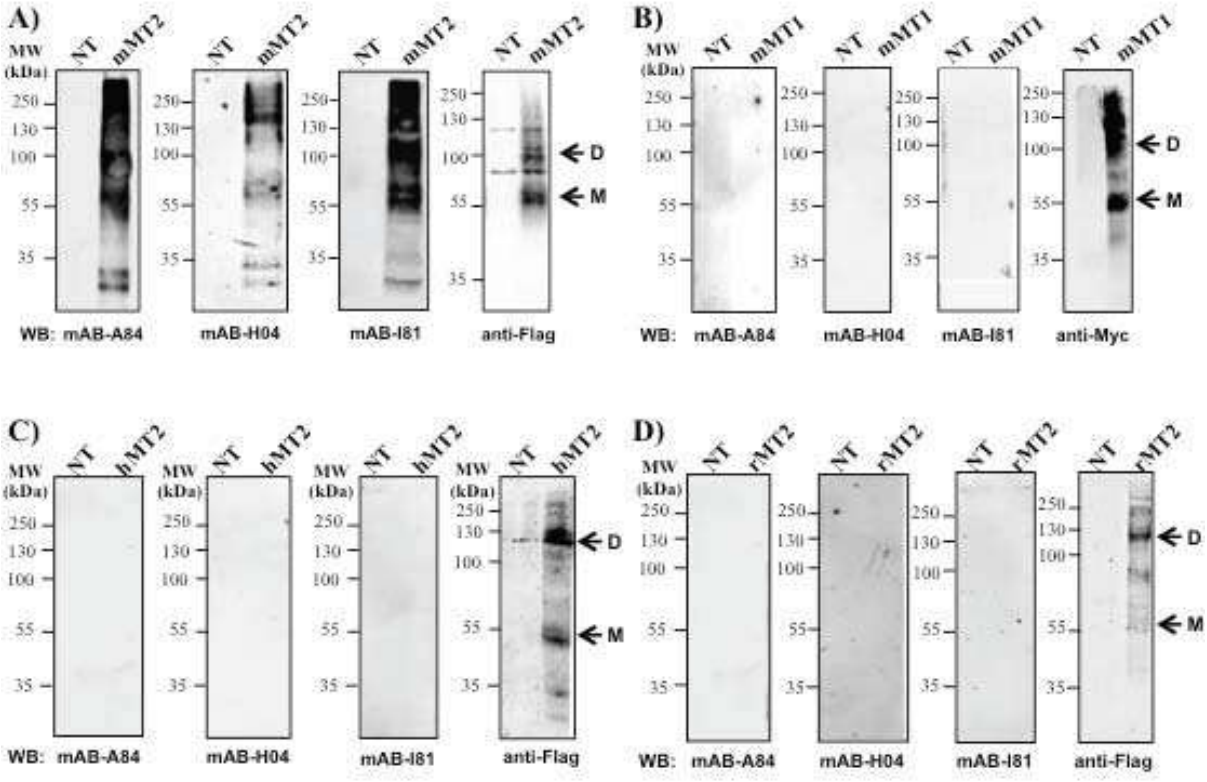

Figure 4

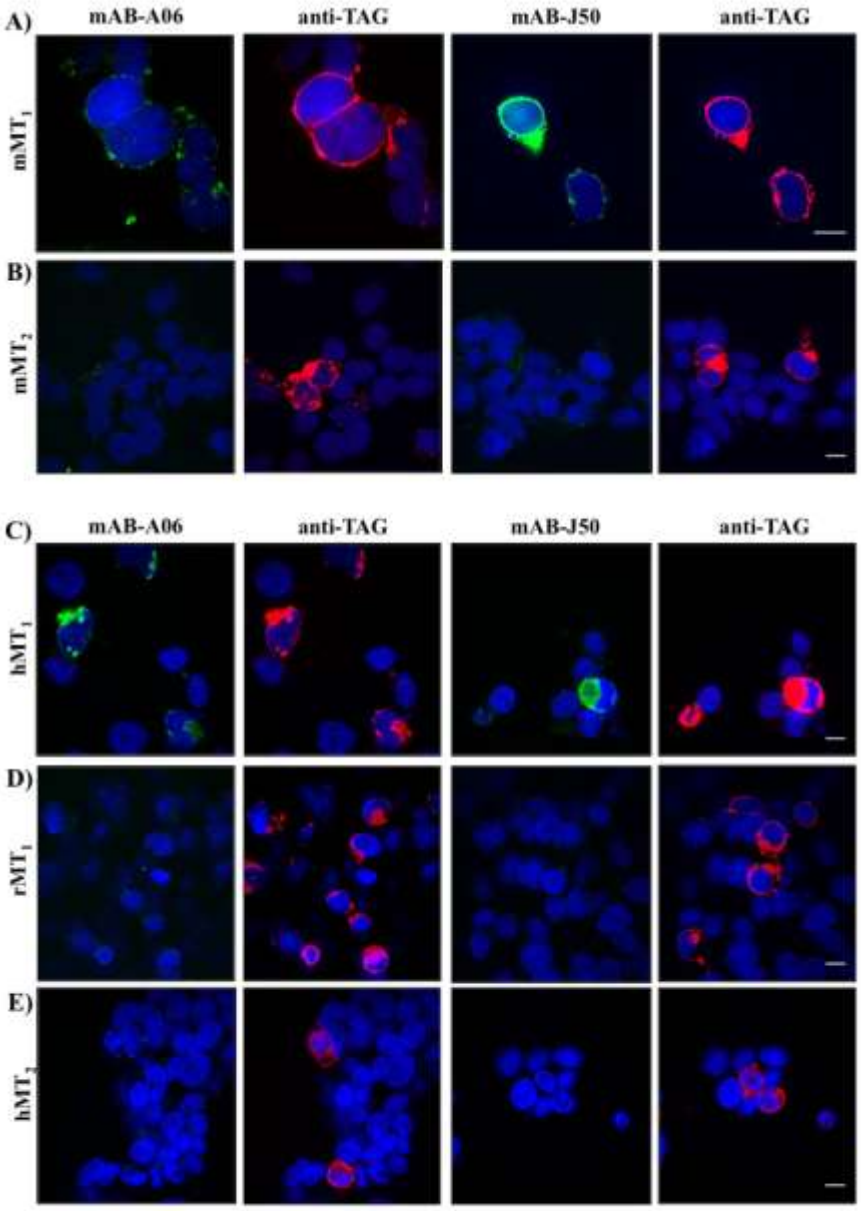

Figure 5

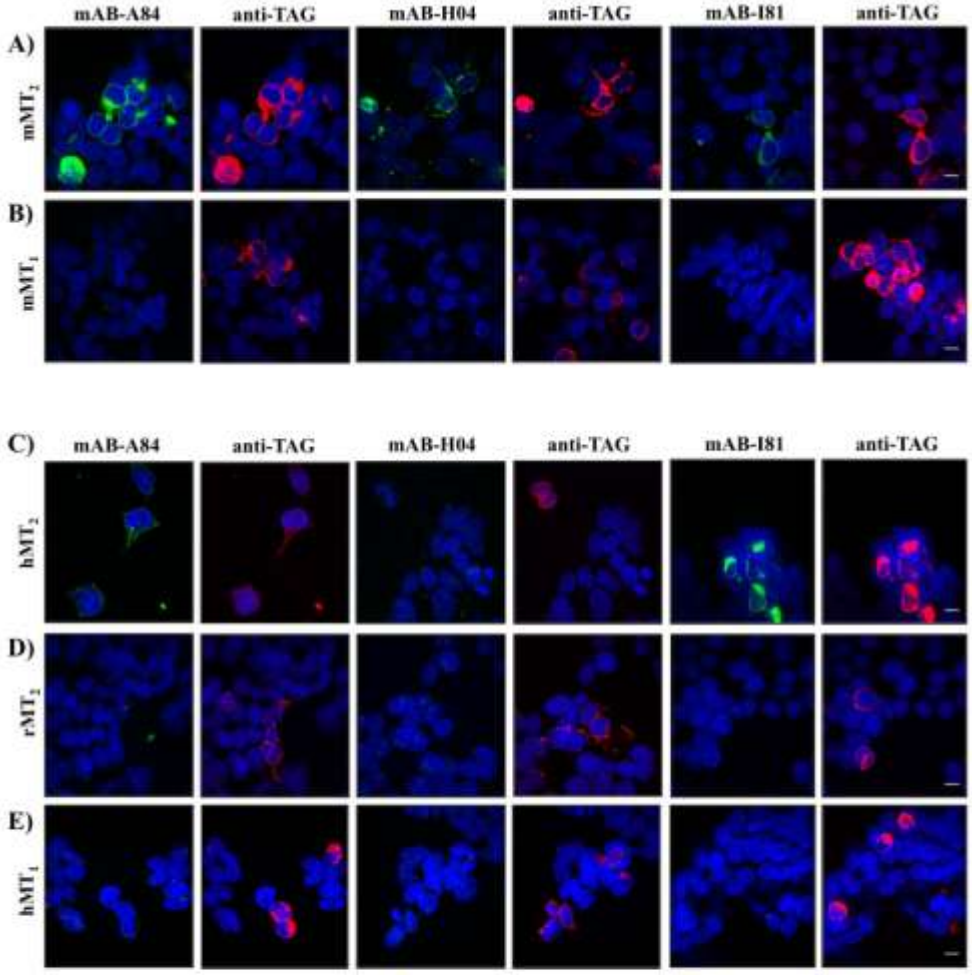

Figure 6

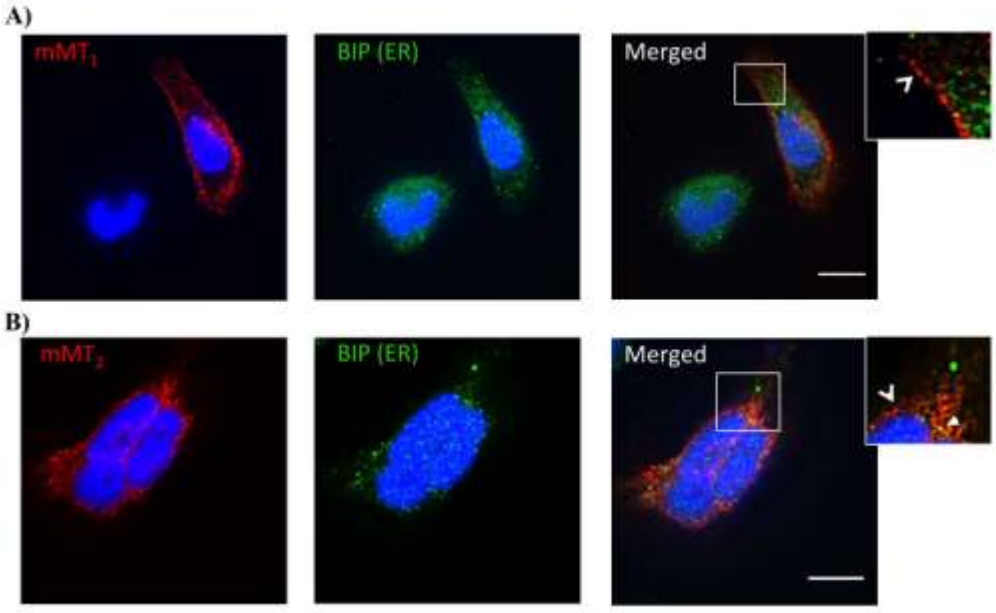

Figure 7

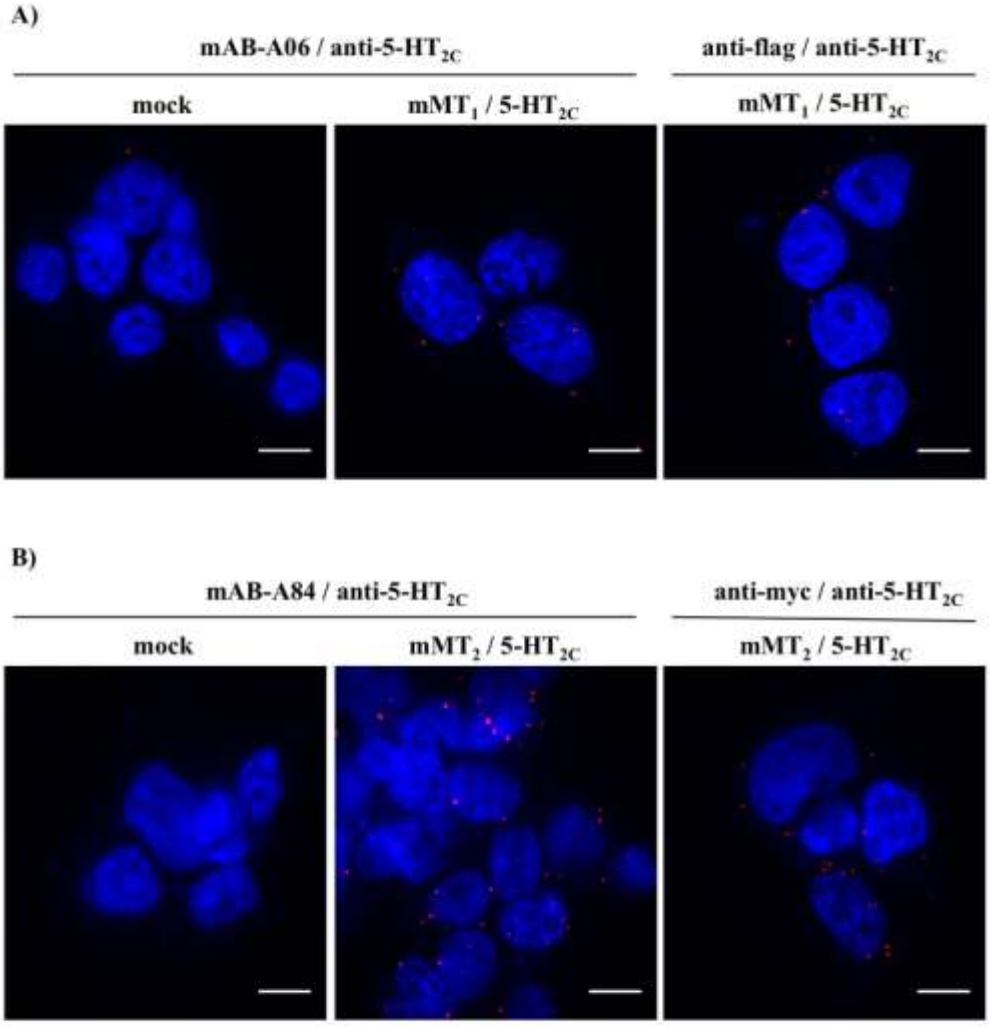

Figure 8

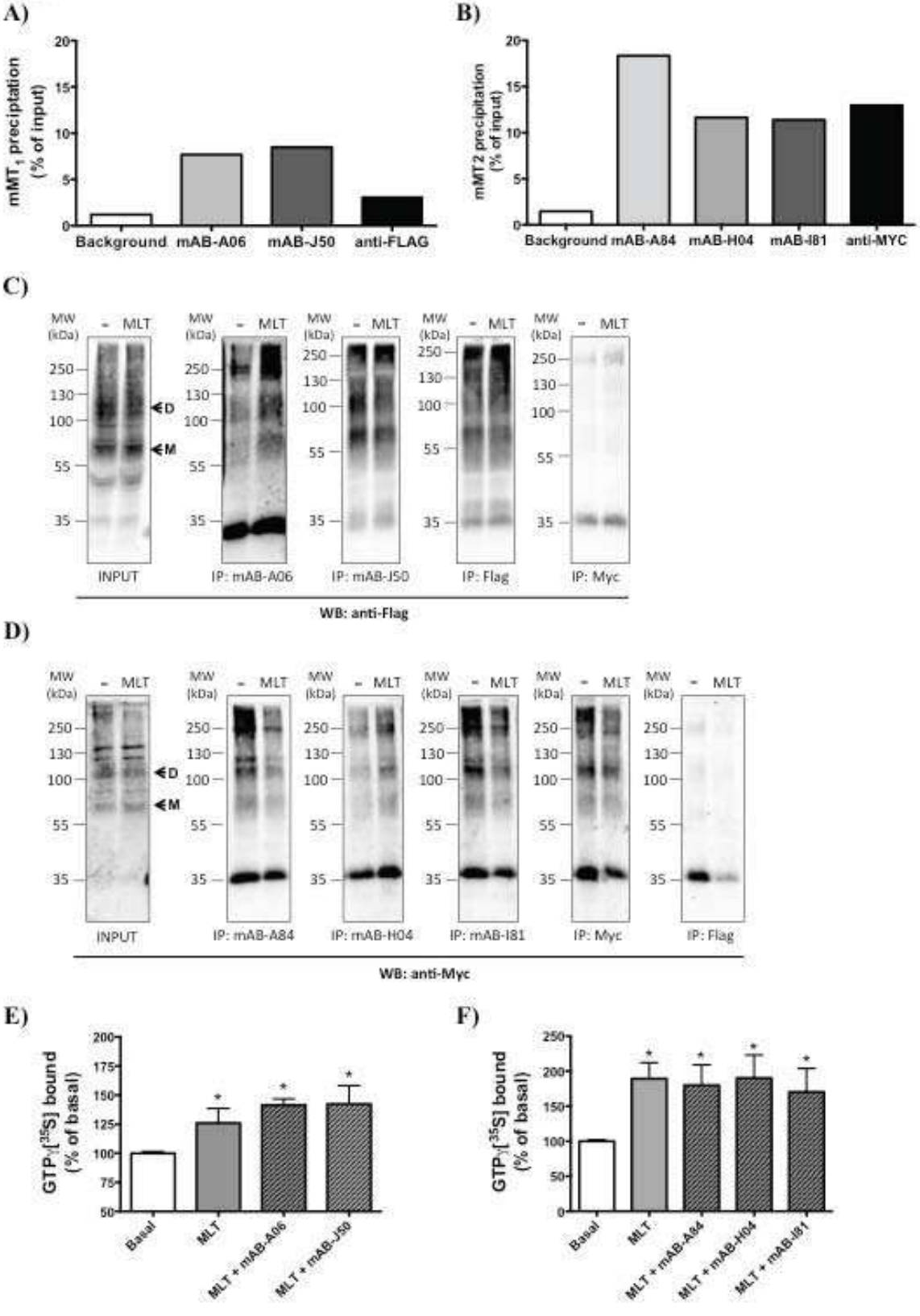

Figure 9