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4 A tagged parathyroid hormone derivative as a carrier of antibody cargoes transported by the G
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6 protein coupled PTH₁ receptor
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4 **Abstract**
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7 Based on the known fact that the parathyroid hormone (PTH) might be extended at its C-terminus
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9 with biotechnological protein cargoes, a vector directing the secretion of PTH₁₋₈₄ C-terminally
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11 fused with the antigenic epitope myc (PTH-myc) was exploited. The functional properties and
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13 potential of this analog for imaging PTH_{1R}-expressing cells were examined. The PTH-myc
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15 construct was recombinantly produced as a conditioned medium (CM) of transfected HEK 293a
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17 cells (typical concentrations of 187 nM estimated with ELISAs for PTH). PTH-myc CM induced
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19 cyclic AMP formation (10 min), with a minor loss of potency relative to authentic PTH₁₋₈₄, and c-
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21 Fos expression (1-3 h). Treatment of recipient HEK 293a cells transiently expressing PTH_{1R} with
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23 PTH-myc CM (supplemented with a fluorescent monoclonal anti-myc tag antibody, either 4A6 or
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25 9E10) allowed the labeling of endosomal structures positive for Rab5 and/or for β -arrestin₁
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27 (microscopy, cytofluorometry). Authentic PTH was inactive in this respect, ruling out a non-
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29 specific form of endocytosis like pinocytosis. Using a horseradish peroxidase-conjugated
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31 secondary antibody, the endocytosis of the PTH-myc-based antibody complex by endogenous
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33 PTH_{1R} was evidenced in MG-63 osteoblastoid cells. The secreted construct PTH-myc represent a
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35 bona fide agonist that support the feasibility of transporting cargoes of considerable molecular
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37 weight inside cells using arrestin and Rab5-mediated PTH_{1R} endocytosis. PTH-myc is also
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39 transported into cells that express PTH_{1R} at a physiological level. Such tagged peptide hormones
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41 may be part of a cancer chemotherapy scheme exploiting a modular cytotoxic secondary antibody
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43 and the receptor repertoire expressed in a given tumor.
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55 Keywords: parathyroid hormone, PTH₁ receptor, osteoblast, receptor-mediated transport.
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List of abbreviations

CM, conditioned medium; mCherry, mCherry fluorescent protein; EGFP: enhanced green fluorescent protein; GPCR, G protein coupled receptor; HRP, horseradish peroxidase; PTH, parathormone; PTH₁R, parathormone receptor 1; **TSA: Tyramide Signal Amplification.**

Introduction

The parathyroid hormone (PTH) is an 84 aminoacid peptide that is a major regulator of bone metabolism and calcium ion handling by tissues via its “class B,” G protein coupled PTH₁ receptor (PTH₁R) expressed in kidneys and the skeleton [14]. Synthetic PTH and its PTH₁₋₃₄ fragment (teriparatide) are currently therapeutically used for an anti-osteoporosis effect [21] and the osteoblast is believed to be their site of action. Furthermore, in a recently reported preclinical model of joint trauma relevant for the physiopathology of osteoarthritis, systemically administered PTH₁₋₃₄ was further proposed to be chondroprotective and chondroregenerative via PTH₁R_s upregulated in chondrocytes [18]. Indeed, PTH is anabolic for cartilage and the serum concentration of the hormone is correlated to cartilage metabolic activity (leucine and sulfate incorporation) in juvenile mice [24]. Further, PTH₁R is expressed in various tumor cells [13,17]. PTH₁R is subjected to agonist-induced, β -arrestin-dependent internalization [9].

Recent crystal structures of agonist-bound PTH₁R extracellular domain revealed that the C-terminal hormone residue does not form direct contacts with the receptor [16]. This made possible two recent biotechnological forms of PTH₁₋₃₄-based agonists elongated at their C-terminus, either with a linker and trans-membrane tether in one case [6,7], or in the other, with the green fluorescent protein (GFP), leading to a high molecular weight probe suitable for imaging studies in cells that express recombinant PTH₁R [3] (schematic representation, Fig. 1A, structure (a)). Here, we evaluated a vector that directs the secretion of the full PTH₁₋₈₄ sequence conjugated at its C-terminus with 2 antigenic tags, including the myc epitope (Fig. 1B), as this construction may be the basis of the modular construction of even larger cargoes based on anti-myc antibodies (Fig. 1A(b-c)). These novel agonist constructs were evaluated for their imaging

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potential in cells expressing recombinant or natural PTH₁Rs. PTH-myc is part of a growing set of myc-tagged agonist peptide hormones that transport antibodies into cells via arrestin-mediated GPCR cycling (bradykinin B₂ receptor [8]; CC-chemokine receptor 7 [4]).

Materials and methods

Cell culture, transfection and analysis

A subclone of HEK 293 cells, called HEK 293a, originally obtained from Sigma-Aldrich was used in most experiments. This cell type was grown in Dulbecco's modified Eagle's medium supplemented with 10 % FBS, 1 % L-glutamine and 1 % penicillin-streptomycin stock solutions (100 ×). The cells were used as recipients of a vector purchased from OriGene Technologies (Rockville, MD): myc-DDK-tagged pro-PTH, that directs the secretion of the mature human PTH₁₋₈₄ sequence extended at its C-terminus with 2 epitopes in tandem, myc and DDK (catalog number RC519848; translated amino acid sequence in Fig. 1). The latter construction will be conventionally designated as PTH-myc. Seventy % confluent producer cells were transfected with a given vector using the ExGen reagent (Fermentas) used as directed.

Other recipient HEK 293a cells were grown and transiently transfected as described above with a vector coding for PTH_{1R} (gift from Dr. T. J. Gardella, Massachusetts General Hospital), and were further optionally co-transfected with mCherry fluorescent protein (mCherry), Rab5-mCherry, Rab5-GTP-locked-mCherry, Rab7-mCherry (given by Dr. M. J. Tremblay, Université Laval, Canada), or β -arrestin₁-mCherry (kind gift from Dr. J.-M. Beaulieu, Université Laval, Canada). Stimulations for microscopic or cytofluorometric experiments were based on the CM of the PTH-myc construction was supplemented with Alexa-Fluor-488-conjugated monoclonal antibodies (clone 4A6, Millipore, dilution 1:1000 corresponding to a final antibody concentration of approximately 3.3 nM in the culture medium). The alternate anti-myc tag monoclonal antibody 9E10 conjugated with Alexa-Fluor-488 was also used in microscopy (Millipore, dilution 1:1000). Cells were generally treated for 30 min with stimulants (incubation carried out at 37°C in humidified atmosphere containing 5% CO₂), rinsed 3 times with phosphate buffered saline, observed in microscopy for epifluorescence and photographed using an Olympus BX51

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4 microscope coupled to a CoolSnap HQ digital camera (filters for AlexaFluor-488: excitation 460-
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6 500 nm, emission 510-560 nm; for mCherry fluorescent protein: excitation 525-555 nm, emission
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8 600-660). The objective lens was generally the 100× oil UPlanApo (Olympus). This system has a
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10 thin focal depth in the relatively thick HEK 293a cells, supporting co-localization studies in a
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12 satisfactory manner. An alternate stimulant was PTH₁₋₈₄ (Sigma-Aldrich). Other transfected cells
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14 were detached using the protease-free Cell Dissociation Buffer (Invitrogen), incubated in D-
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16 MEM without serum at 37°C for 30 min under agitation in the presence of a stimulant, rapidly
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18 centrifuged (30 sec, 11,000 g) and resuspended in phosphate buffered saline. Then, the
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20 fluorescence of the cell suspensions was assessed using the BD SORP LSR II cell analyzer for
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22 the uptake of a green fluorophore as a function of stimulation and transgene expression.
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32 *Detection of endogenously expressed PTH_{1R} using the endocytosis of PTH-myc-anti-myc*
33 *antibody complexes*
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37 The antibody cargo system has been adapted to detect PTH_{1R}-mediated transport in relevant cells
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39 and also in HEK 293a expressing recombinant PTH_{1R}, for comparison. The human osteoblastoid
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41 cells MG-63, originally obtained from the ATCC and propagated in α -MEM supplemented with
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43 10% fetal bovine serum, antibiotics and L-glutamine, was also used in some experiments. Cells
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45 have been incubated for 30 min (37°C) in the PTH-myc or control CM supplemented with the
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47 non-labeled 4A6 monoclonal antibody (Millipore, final concentration \approx 3.3 nM). Then, cells were
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49 fixed, permeabilized and stained with the Tyramide Signal Amplification (TSA) Kit containing
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51 horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibodies and AlexaFluor-488-
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53 tyramide as a fluorogenic co-substrate of the reaction (Invitrogen kit T20912 used as directed).
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59 **The TSA technology applied to immunohistochemistry is reviewed elsewhere [23].**
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7 *Immunoblots*
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9 The agonist action of PTH-related agonists was investigated using the expression of the
10 transcription factor c-Fos, a distal signaling response to the stimulation of various receptor-ligand
11 systems [10]. Total HEK 293a cell extracts were immunoblotted to detect c-Fos expression using
12 the K-25 rabbit polyclonal antibodies (Santa Cruz Biotechnology; dilution 1:100).
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21 *ELISA of cyclic AMP and PTH*
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23 A commercial cyclic AMP ELISA kit (Cell Biolabs, San Diego, CA) was applied as directed
24 without the optional acylation reaction to quantify the intracellular second messenger of PTH_{1R}-
25 expressing HEK 293a cells (confluent 35-mm petri dishes) variously stimulated. Another ELISA
26 kit, the Intact Parathyroid Hormone ELISA Kit (GenWay Biotech, San Diego, CA) was used to
27 quantify PTH-myc in CM (dilution 1:10 – 1:200). This assay sequentially exploits antibodies
28 directed to both the N- and C-terminal regions of human PTH₁₋₈₄ and, therefore, is selective for
29 the whole sequence peptide.
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43 *Data analysis*
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45 Numerical values are reported as means \pm s.e.m. Non-normally distributed groups of values were
46 analyzed using nonparametric analysis of variance (Kruskal-Wallis test) followed by Dunn's
47 multiple comparison test. Normal sets of values were compared using ANOVA followed by
48 Tukey-Kramer multiple comparison test or, for comparison with a common control value,
49 Dunnett's test (InStat 3.05 computer program, GraphPad Software; San Diego, CA).
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Results

Characterization of PTH-myc as carrier of an antibody cargo: visualization tool

The CM of HEK 293a cells transiently transfected with the vector coding for the tagged protein PTH-myc contained the equivalent of 187 ± 15 nM of PTH (ELISA for full sequence PTH, n = 3). The control CM of untransfected cells did not contain measurable immunoreactive PTH (n = 2). In acute tests (10-min stimulation), PTH-myc was about 4-fold less potent than PTH₁₋₈₄ to raise cyclic AMP in HEK 293a cells that expressed recombinant PTH_{1R} (ELISA determinations, Fig. 2). The c-Fos expression and extensive phosphorylation (detected as an increasing ladder of immunoreactive proteins) induced by PTH-myc CM were much more intense than that produced by 10 nM synthetic PTH₁₋₈₄ (Figure 3), consistent with the higher immunoreactive hormone concentration in the CM. The fact that multiple bands are detected with the anti-cFos antibody may also derive from certain cross-reactivities with the c-Fos homologues Fos B and Fra-2, according to the antibody manufacturer. c-Fos signaling was persistent, as both forms of stimulation had declining but detectable effects after 12 h. In experiments reported in Figures 2 and 3, synthetic PTH₁₋₈₄ was added to the serum-containing culture medium, thus supporting reasonable comparisons with PTH-myc present in the CM.

Experiments were designed to evidence the PTH_{1R}-mediated cellular uptake of immune complexes formed in the culture medium and composed of PTH-myc bound to a fluorescent anti-myc antibody (microscopy, Figure 4, or cytofluorometry, Figure 5). Controls included cells that did not express PTH_{1R}, cells stimulated with synthetic PTH₁₋₈₄ (10-100 nM) or with the CM of untransfected HEK 293a cells. A very robust and specific endosomal labeling of recipient cells that expressed the receptor was observed (Figure 4). These observations support that the PTH-

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4 myc-4A6 (Fig. 4A) or PTH-myc-9E10 immune complexes (Fig. 4B) formed in the culture
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6 medium were recognized by the PTH₁R as agonists and were transported into endosomes.
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8 Cytofluorometry confirmed these results (based on the fluorescent 4A6 antibody, Figure 5). Of
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10 note, synthetic PTH₁₋₈₄ devoid of the myc epitope was not competent to induce the endocytosis
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12 (pinocytosis) of droplets of the culture medium containing the fluorescent antibody (Figures 4
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14 and 5), further supporting the specific molecular interaction of the myc-tagged agonist with the
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16 antibody. Receptor-expressing cells co-treated with the PTH-myc-4A6 immune complexes and
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18 with synthetic PTH₁₋₈₄ (150 nM or 1.52 μM) evidenced concentration-dependent competition for
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20 endocytosis of the fluorescent antibody by an excess of the unlabeled agonist (Fig. 4C).
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29 The PTH-myc/4A6 costimulation scheme supported β-arrestin₁ colocalization studies (Figure 6,
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31 top). The mCherry-tagged arrestin is homogeneously expressed in the cytosol of recipient cells
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33 that co-expressed PTH₁R. Both PTH₁₋₈₄ and the CM of PTH-myc induced the condensation of β-
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35 arrestin₁-mCherry into endosomal structures, but only the myc-tagged agonist carried with it the
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37 fluorescent antibody that was extensively colocalized with condensed β-arrestin₁-mCherry
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39 (Figure 6). Thus, tetramolecular complexes must be detected at the endosomal level: antibody-
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41 agonist-receptor-arrestin, with the first and fourth molecules only being fluorescent.
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46 Colocalization of the PTH-myc-fluorescent anti-myc antibody complex was also tested in cells
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48 expressing the receptors and one of two Rab5-mCherry constructions: the GTP-locked activated
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50 form that causes the formation of giant endosomes [19] or the wild type one (Figure 7):
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52 colocalization was extensive in cells treated for 30 min with the CM of PTH-myc. Rab5 is a
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54 marker of early endosomes, whereas Rab7 rather labels late endosomes. While the PTH-myc/4A6
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56 did not colocalize with Rab7-mCherry after 30 min of incubation, minor colocalization was
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4 observed after 3 or 6 h (Figure 8), indicating a slow progress of the antibody cargo in the
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6 endosomal/lysosomal tract.
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10 *Application of PTH-myc to imaging of endogenously expressed PTH₁R*

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12 In HEK 293a cells that express PTH₁R, but not in control cells, a very bright intracellular signal
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14 was detected following endocytosis of the PTH-myc-4A6 antibody complex using the TSA
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16 system that enzymatically generates AlexaFluor-488 labeling (Figure 9A). This amplified
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18 detection system has been applied to detect the naturally expressed PTH₁R in relevant cells.
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26 The human osteoblastoma MG63 cell line is known to express PTH₁R and to functionally
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28 respond in several ways to PTH₁₋₈₄, PTH₁₋₃₄ and other ligands [5,11,12,25]. This cell line exhibits
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30 a slight but consistent granular uptake of the AlexaFluor-488-conjugated anti-myc antibody if co-
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32 treated with PTH-myc CM, but not when exposed to the control CM of HEK 293a cells
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34 (microscopy, Figure 9B). The enzymatically amplified TSA detection system, based on a HRP-
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36 conjugated secondary antibody, revealed the endocytosis of the 4A6 antibody-PTH-myc complex
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38 in a more intense manner with some non-specific nuclear staining (Figure 9B). These
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40 experiments establish that the same hierarchy of sensitivities for PTH₁R visualization techniques
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42 exists in MG-63 cells as in HEK 293a cells that express recombinant receptors, but lower
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44 intensities in the system that expresses low physiological levels of PTH₁R.
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Discussion

Because the secretin receptor family is tolerant to variations in the C-terminal structure of peptide ligands [7], we hypothesized that PTH-myc, efficiently secreted and combined in the extracellular milieu with an anti-myc antibody, would support the endocytosis of a very large agonist cargo (in excess of 150 kDa) mediated by recombinant or naturally expressed PTH_{1R} (Figures 4-10). PTH-myc is a close analog of the full hormone sequence and both increased cellular cyclic AMP (Fig. 2) and exert a prolonged effect on the c-Fos expression assay (Figure 3). The recombinant peptide PTH-myc has been compared to synthetic PTH₁₋₈₄ in the assay of acute cyclic AMP generation (Fig. 3), but not in the slow c-Fos induction assay (Fig. 4), where arbitrary agonist concentrations were used; the former assay evidence only a minor loss of potency in the myc-tagged agonist. Findings suggest that binding of the tagged agonist to the antibody does not impair the recognition of the receptor by PTH₁₋₈₄ and that this design enables protein cargoes even larger than green fluorescent protein [3] to be transported by the activated PTH_{1R}. The anti-myc 4A6 monoclonal antibody used in the present work has previously been shown to be internalized by the activated bradykinin B₂ receptors, whether the myc epitope was located in the receptor construction or at the N-terminus of a synthetic agonist [2,8], and also by the chemokine receptor CCR7 via a myc-tagged agonist (CCL19-myc) [4]. The alternate 9E10 anti-myc monoclonal antibody was also internalized as a cargo tightly bound to the agonist PTH-myc, despite its more unpredictable behavior vs. sequences that flank the myc epitope in protein constructions [1]. We have not exploited the built-in DDK epitope present in the commercial PTH-myc construction (Fig. 1), because a typical commercial anti-DDK tag is both less sensitive and specific than the anti-myc monoclonals in our hands. Importantly, results highlight that detection of the co-endocytosed 4A6 monoclonal antibody using an enzymatically amplified

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4 reaction is a powerful visualization aid for the PTH₁R that proved cargo endocytosis by
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6 endogenously expressed receptors in the osteoblastoid cells MG-63.
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11 The anti-osteoporosis peptide PTH₁₋₃₄ is inactivated by receptor-expressing cells and has a short
12 duration of action in-vivo; daily administration of this agent leads to a “pulsed” effect on
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14 osteoblast that is believed to be obligatory for the desired anabolic effect [22]. The PTH-myc
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16 construction is based on the PTH₁₋₈₄ sequence known to undergo massive receptor-mediated
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18 endocytosis and that determines slow intracellular cycling relative to the alternate agonist PTH-
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20 related peptide (PTHrP) [22]. Further highlighting the complexity of this system, recent evidence
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22 supports that non-canonical signaling emerging from endocytosed PTH₁Rs bound to β-arrestins
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24 may also impact the relative therapeutic efficacy of these analogs [9,22]. These observations may
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26 reflect different cycling and signaling kinetics relative to the parent peptides. Although beyond
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28 the scope of the present study (focused on imaging applications), such future analyses will be
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30 important to better understand how heavy C-terminal modifications impact the pharmacological
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32 properties of emerging class B GPCR agonist therapeutics. For instance, albiglutide is a new anti-
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34 diabetic glucagon-like peptide 1 receptor agonist fused to albumin; this fusion protein exhibits
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36 considerable alteration of pharmacokinetics relative to the parent peptide [20].
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48 Altering the cargo protein at the C-terminus of the PTH₁R activating sequence could lead to more
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50 ambitious applications that are not currently supported by the present study. (1) In relationship to
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52 the recently proposed use of a systemically administered PTH₁R agonist in osteoarthritis [18], the
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54 present study suggests the possibility to produce alternate high molecular weight agonists adapted
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56 to intra-articular injection with decreased systemic diffusion, prolonged signaling and decreased
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58 “off target” side effects. (2) Further, a functional cytotoxic protein cargo conjugated to PTH
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4 could theoretically be useful to treat malignant chondrosarcomas, where PTH₁R expression is
5 consistently upregulated in a manner correlated with histological grade [17]. PTH₁R is also
6 expressed in an apparently ectopic manner in a number of human carcinomas (e.g., prostate,
7 pancreas) [13]. In such application, a novel generation of anti-myc monoclonal antibodies armed
8 with cytotoxic drugs via acid-labile bonds that are released in endosomes [15] could be targeted
9 to tumor cells that express PTH₁R (as modeled in MG-63 cells). The strategy is modular, being
10 adaptable to other tagged agonists, for instance CCL19-myc, an agonist of CCR7 often expressed
11 in metastatic tumors [4]. The agonist effect of the peptide hormone vector, documented for PTH-
12 myc, would not necessarily be detrimental because the transient stimulation of tumor cell mitosis
13 or metabolism could selectively increase their sensitivity to anti-mitotic drugs conjugated to the
14 secondary antibody.
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33 Possessing an intact N-terminal receptor-binding sequence, the construction PTH-myc is a *bona*
34 *fide* bifunctional agonist that shows the feasibility of the transport of cargoes of considerable
35 molecular weight by PTH₁R. The sensitive detection of PTH₁R using the tagged agonist, primary
36 anti-tag antibody and secondary HRP-conjugated antibody may rivals the immunohistochemistry
37 based on an anti-PTH₁R monoclonal antibody [13], but is potentially applicable to multiple
38 animal species, because the agonist effect of peptide hormones may cross the species barrier
39 more easily than the reactivity of anti-receptor antibodies. Considering the structural similarities
40 between class B1 GPCR hormones, it may be anticipated that additional peptides belonging to
41 this family will show useful pharmacologic properties when conjugated to antigenic tags for
42 imaging purposes or tissue-specific delivery of biotechnological cargoes.
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15 equipment and Dr. Alexandre Brunet for operating the cytofluorometric equipment.
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4 **Figure legends**
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9 Figure 1. A. Schematic representation of the PTH₁R and its bifunctional agonist ligands that carry
10 cargoes of increasing molecular masses. (a) PTH₁₋₃₄-EGFP [3]; (b) and (c) PTH₁₋₈₄ tagged with
11 the myc epitope in a C-terminal extension, adapted to transport anti-myc antibody-based cargoes.
12 AF488: AlexaFluor-488 fluorophore; HRP: conjugated horseradish peroxidase. B. Amino acid
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Figure 2. Cyclic AMP production in petri dishes of HEK 293a transiently expressing PTH₁R and acutely (10-min) stimulated as indicated. Results are means of duplicate determinations in a representative experiment out of a set of two.

Figure 3. Induction of c-Fos in HEK 293a cells expressing or not PTH₁R in response to treatments with conditioned media relevant for PTH-myc or PTH₁₋₈₄. Results representative of 2 experiments.

Figure 4. Endocytosis of anti-myc monoclonal antibodies as determined by co-treatment with the PTH-myc construction in HEK 293a cells that optionally and transiently expressed PTH₁R. The undiluted conditioned media supplemented with antibodies were transferred for a 30-min incubation period at 37°C before rinsing and observation. A. Anti-myc clone 4A6, conjugated to AlexaFluor-488, final concentration in the culture medium 3.3 nM. B. Alternate anti-myc 9E10 clone similarly conjugated. In A and B, control conditioned medium or PTH₁₋₈₄ were used as control stimuli. C. Competition of the endocytosis of the PTH-myc-4A6 immune complexes by co-treatment with unlabeled PTH₁₋₈₄. Original magnification 1000 ×.

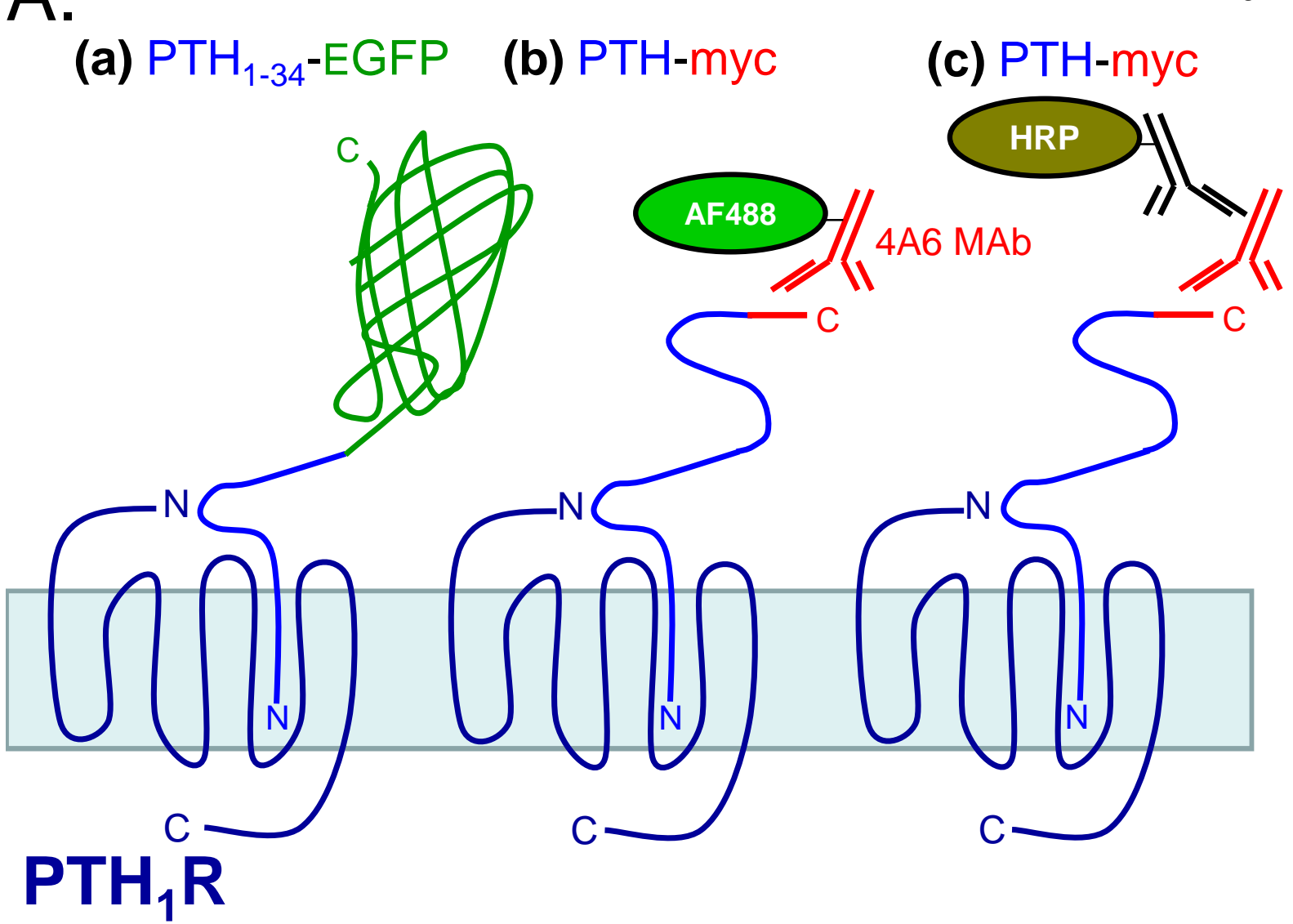
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7 Figure 5. Cytofluorometry of HEK 293a cells that optionally expressed PTH₁R stained with the
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9 AlexaFluor-488-conjugated anti-myc monoclonal antibody 4A6. The antibody was added to the
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11 culture medium of intact cells along with the indicated co-treatment, undiluted conditioned
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13 medium of other cells, some producing PTH-myc, or PTH₁₋₈₄. Left: distributions based on the
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15 counting of 10000 cells. A threshold of autofluorescence was defined using cells treated with the
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17 control conditioned medium. It was surpassed only under one set of experimental conditions
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19 (arrow). Right: proportion of cells above the threshold under each experimental condition in 3
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21 separate experiments (means ± s.e.m.). ANOVA indicated that the values were heterogeneous
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23 (P<0.01). * P<0.01 vs. controls with or without receptors (Tukey-Kramer multiple comparison
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25 test).
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34 Figure 6. Colocalization studies in HEK 293a cells expressing PTH₁R and mCherry-conjugated
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36 β-arrestin₁. The green signal derives from the endocytosis of the AlexaFluor-488-conjugated anti-
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38 myc monoclonal antibody 4A6. Cells were stimulated as indicated for 30 min. Epifluorescence,
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40 original magnification: 1000 ×.
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46 Figure 7. Colocalization studies in HEK 293a cells expressing PTH₁R and mCherry-conjugated
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48 Rab5 or the GTP-locked variant of the latter. Presentation as in Figure 6.
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54 Figure 8. Colocalization studies in HEK 293a cells expressing PTH₁R and mCherry-conjugated
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56 Rab7. Cells were stimulated for variable durations with the conditioned medium of other cells
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58 producing PTH-myc. Presentation as in Figure 6.
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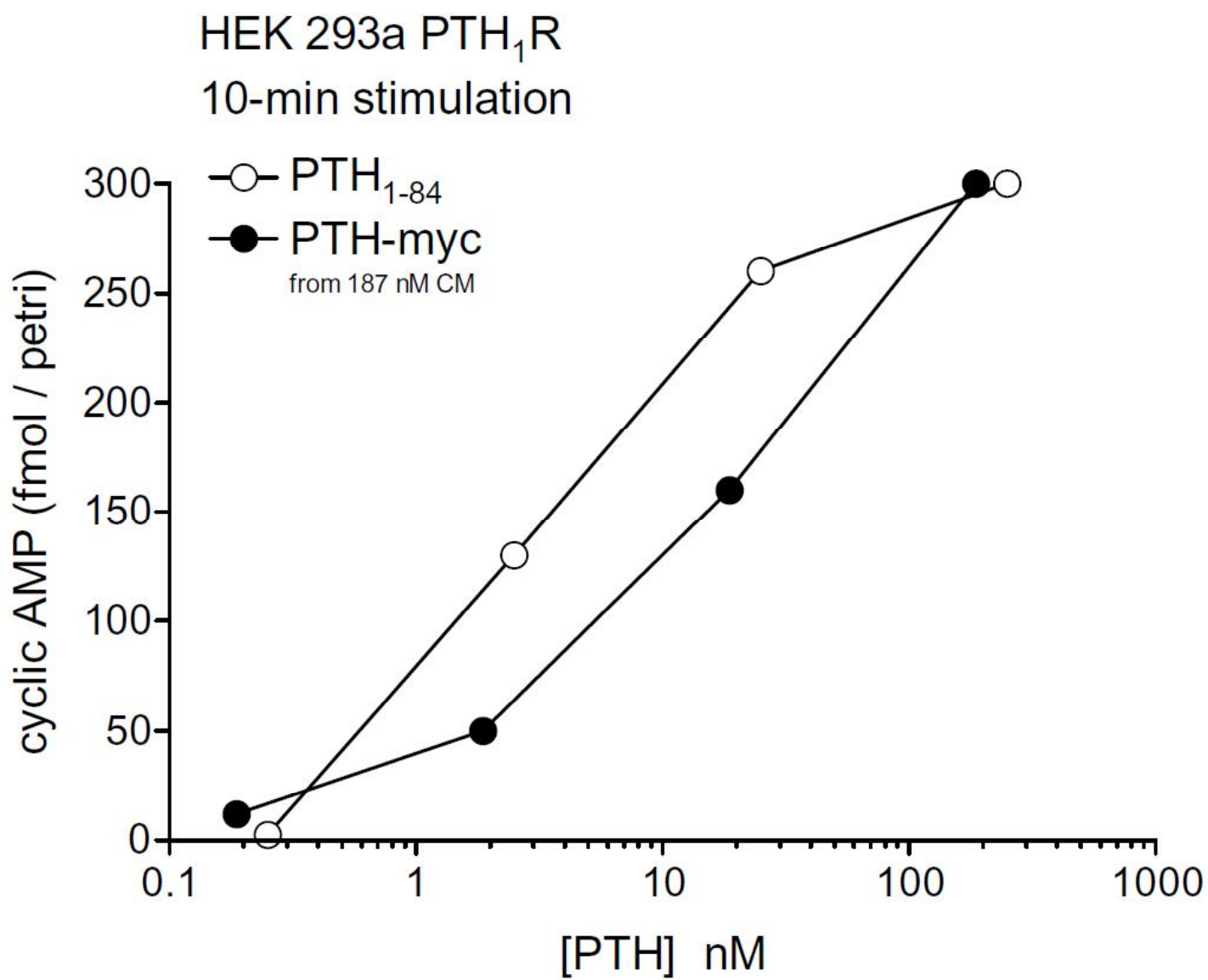
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7 Figure 9. Detection of recombinant or endogenous PTH₁R using detection of the endocytosed
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9 PTH-myc-4A6 antibody complex using the Tyramide Signal Amplification (TSA) system that
10 enzymatically generates AlexaFluor-488 labeling. A. Verification with HEK 293a cells that
11 optionally express the recombinant receptor and were treated with the PTH-myc CM along with
12 the non-fluorescent 4A6 antibody (~3.3 nM). B. Application to MG-63 osteoblastoid cells and
13 comparison of the TSA detection with the cell green autofluorescence and to the fluorescence
14 associated with endocytosed AlexaFluor-488-conjugated anti-myc antibodies. Epifluorescence
15 and transmission, original magnification: 1000 ×.
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B. PTH-myc =
prepro-PTH₁₋₈₄, human, tagged (OriGene)

*MIPAKDMAKVMIVMLAICFLT*KSDGKSVKKR
 SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNFVALGAPLAP
 RDAGSQRPRKKEDNVLVESHEKSLGEADKADVNVLTAKASQ
 TRTRPLEQKLISEEDLAANDILDYKDDDDKV_{stop}

31 first residues = signal peptide
84 next residues = mature PTH
cloning sites + joining peptides
myc tag
DDK tag
 mature protein 13.0 kDa



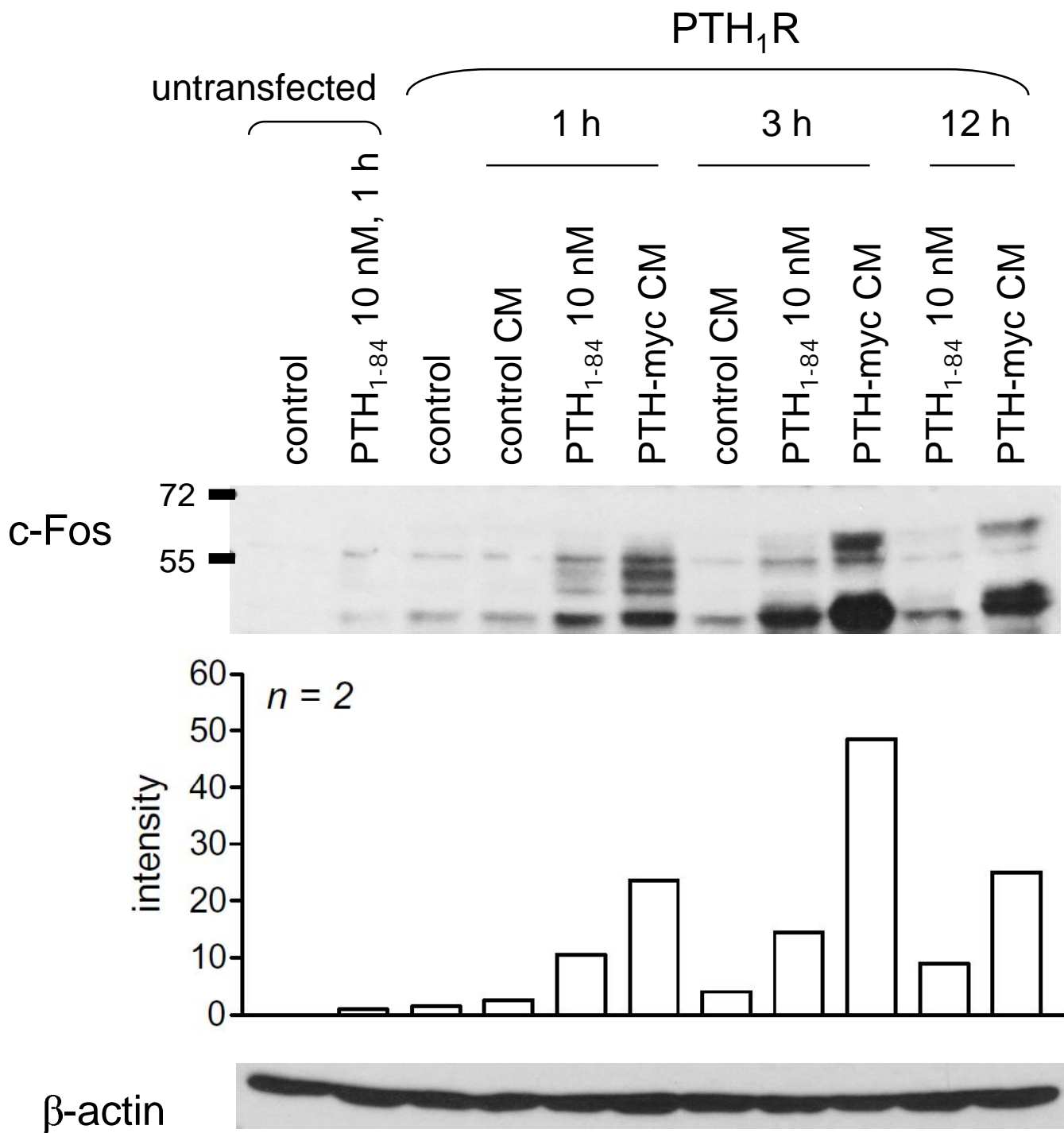


Figure 4

recipient HEK 293a cells

A. 4A6-AF488 +

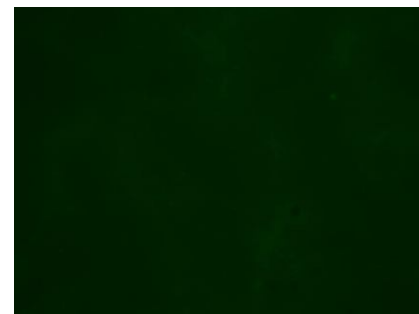
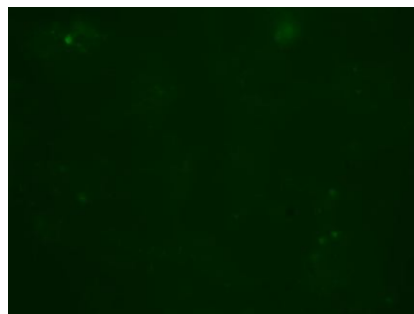
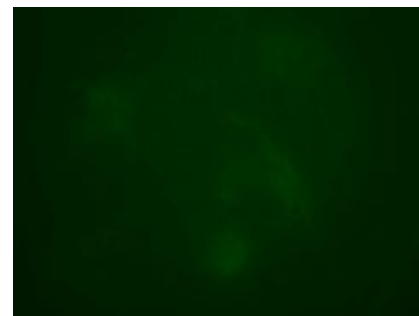
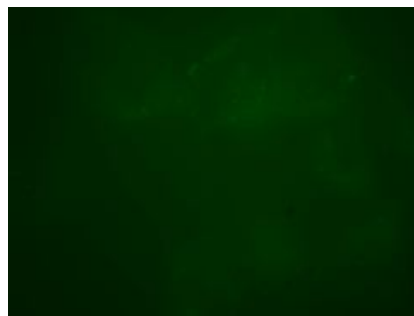
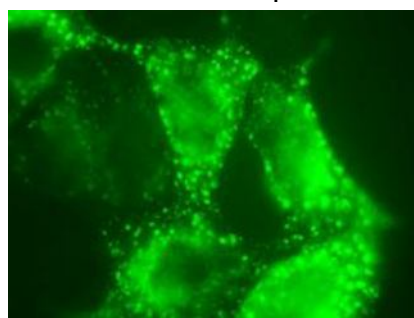
PTH-myc CM

control CM
(HEK 293a)

PTH₁₋₈₄
100 nM

PTH₁R

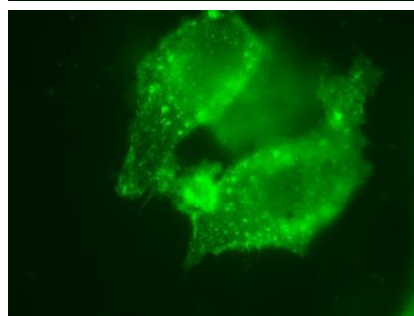
untransfected



B. 9E10-AF488 +

PTH-myc CM

control CM
(HEK 293a)



**C. PTH₁R +
PTH-myc CM +
4A6-AF488 +**

control

PTH₁₋₈₄ 150 nM

PTH₁₋₈₄ 1.5 μM

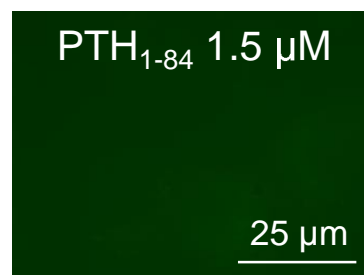
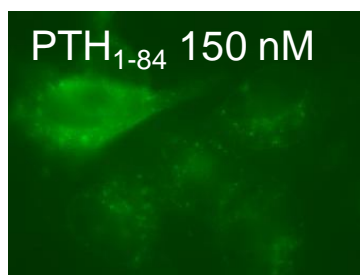
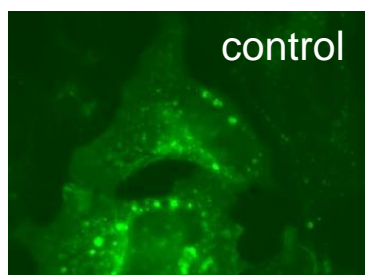


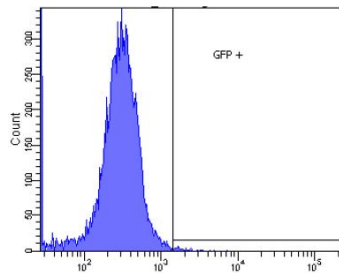
Figure 5

Fig. 5

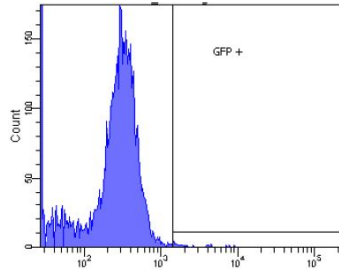
4A6-AF488 +

HEK 293a

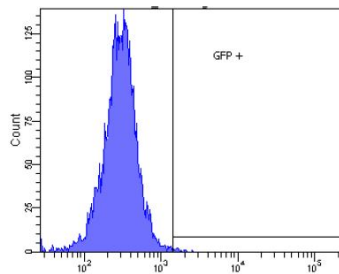
control CM



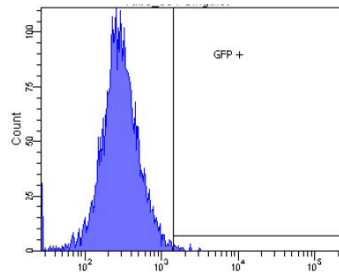
PTH-myc CM



PTH₁₋₈₄ 10 nM

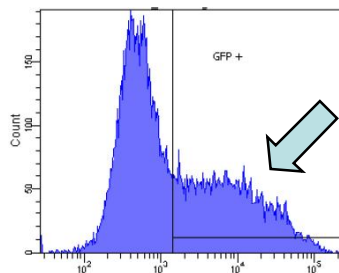


control CM



**HEK 293a
+ PTH_{1R}**

PTH-myc CM



PTH₁₋₈₄ 10 nM

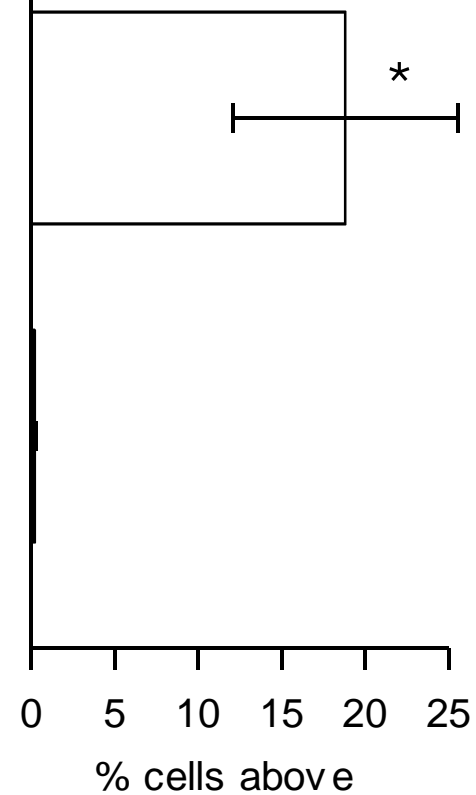
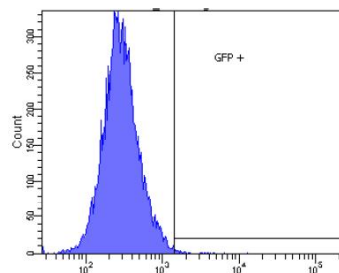


Figure 6

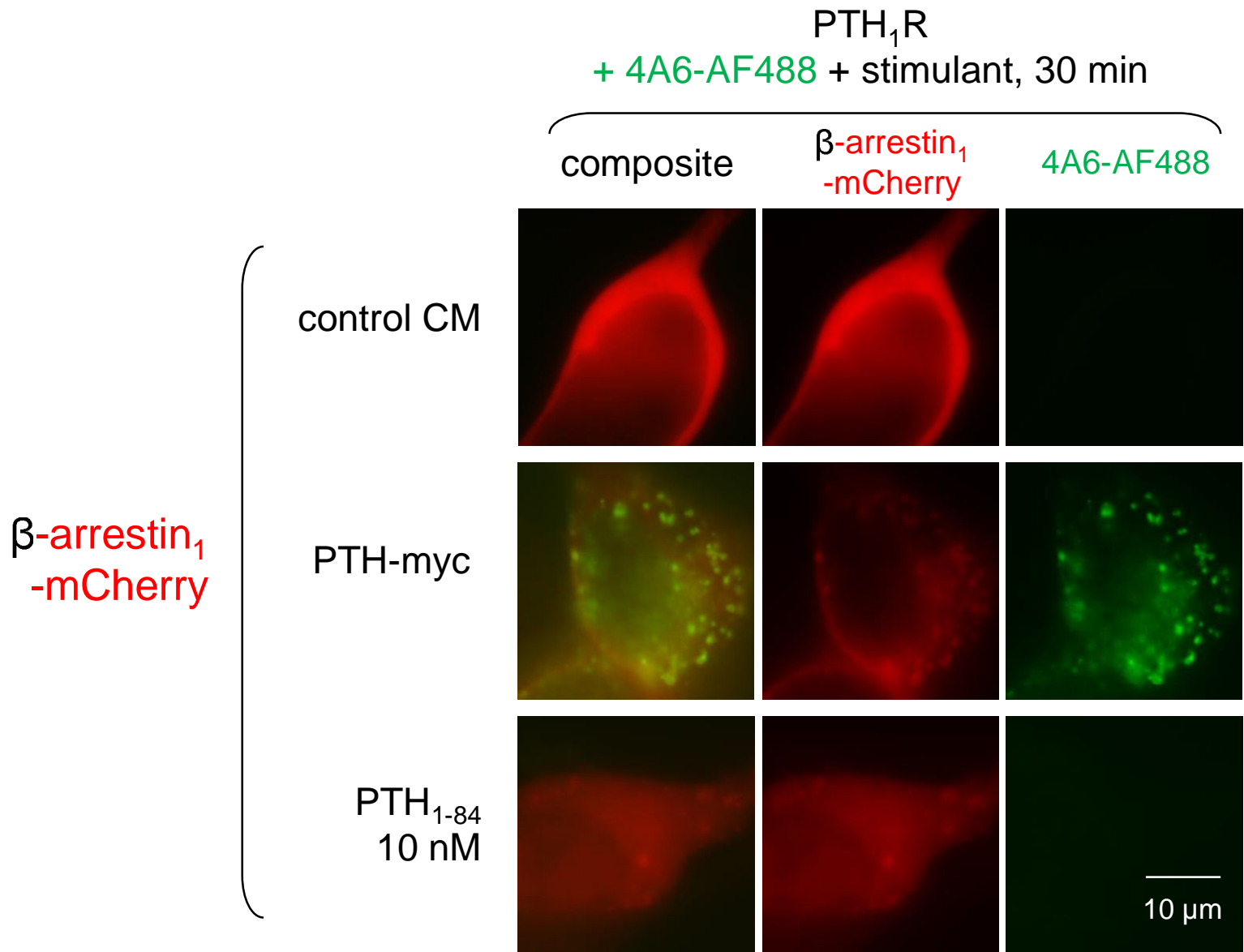


Figure 7

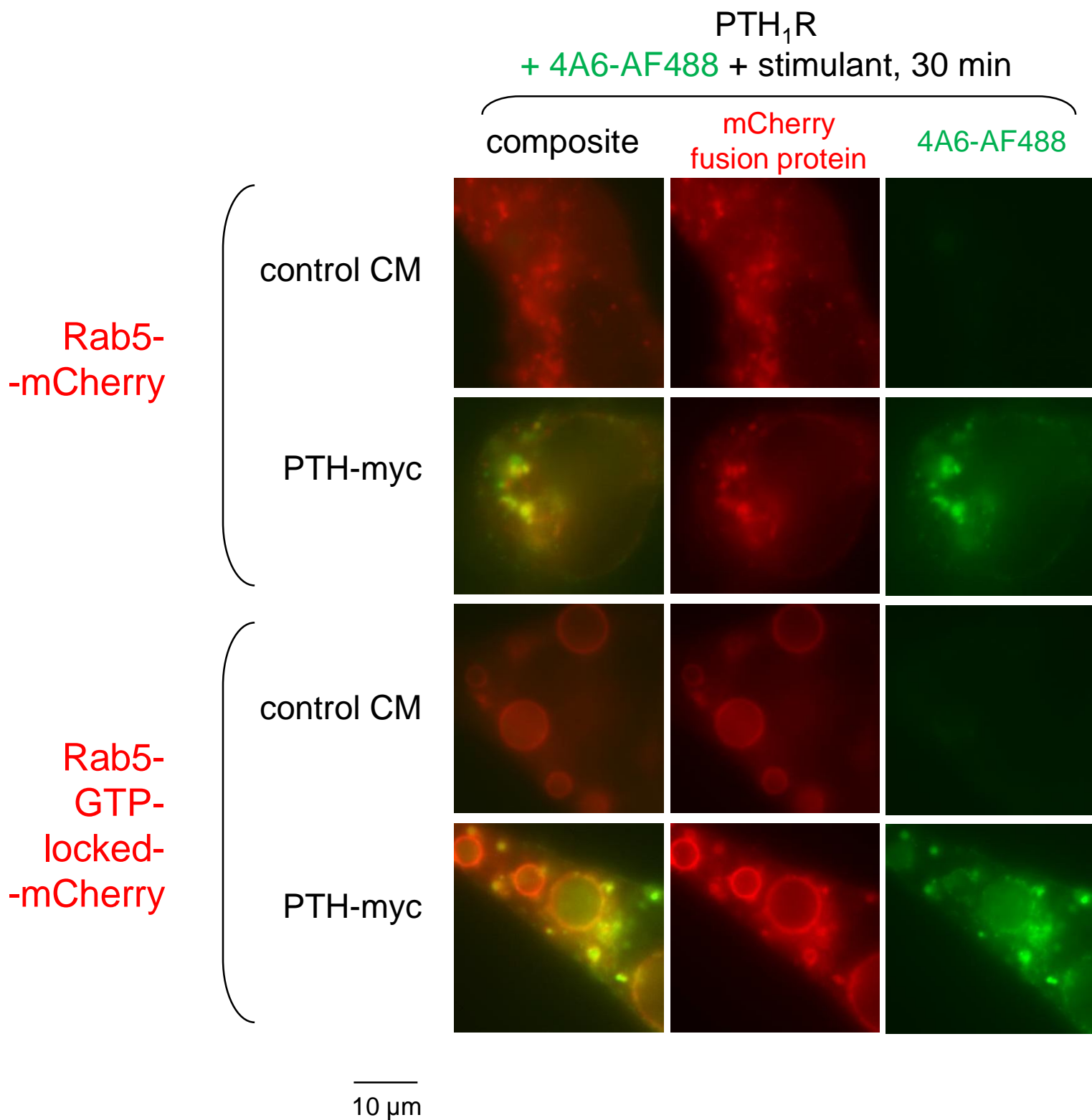
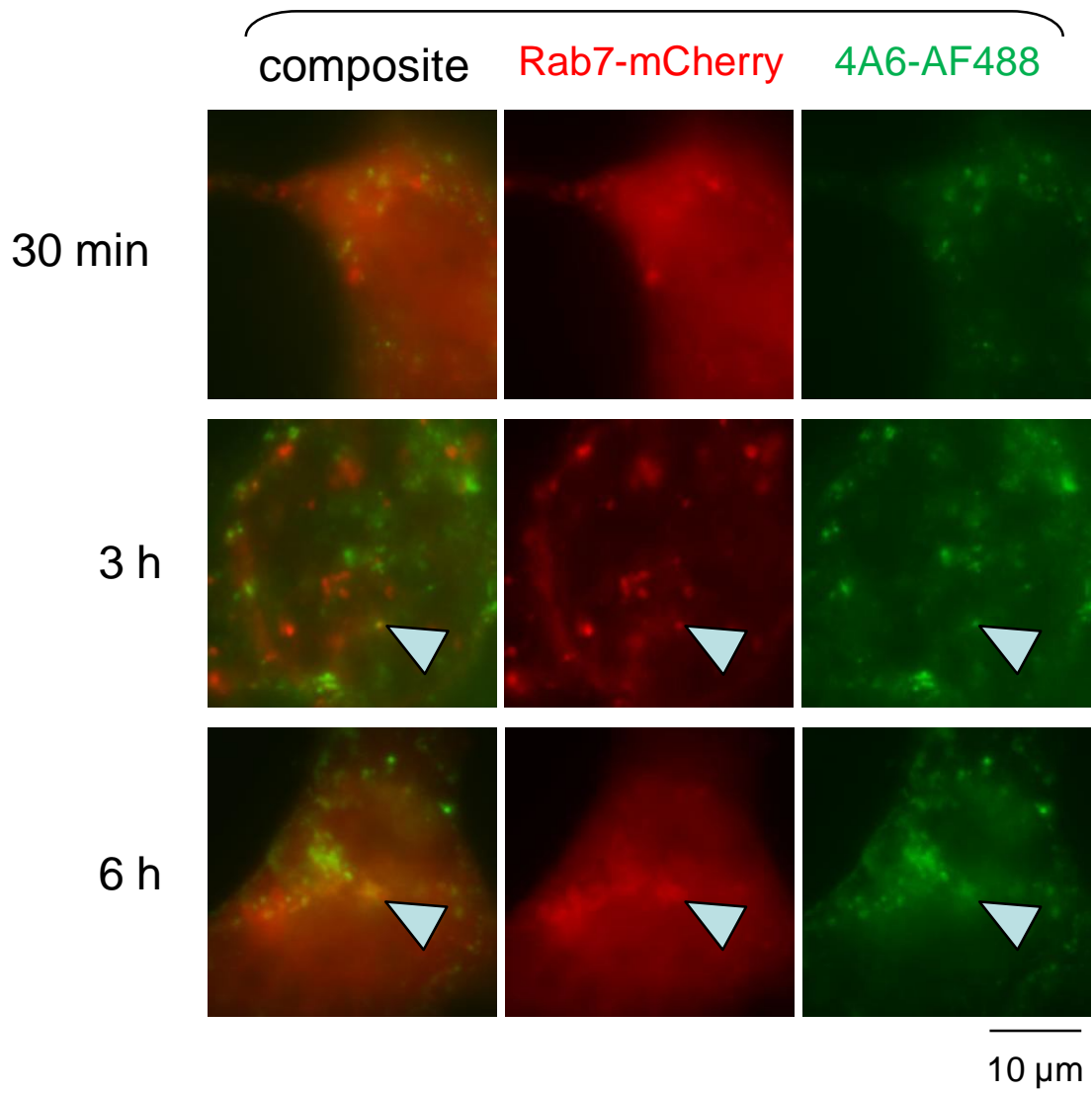


Figure 8

PTH₁R + Rab7-mCherry
+ 4A6-AF488 + PTH-myc



HEK 293a: 4A6 + PTH-myc CM

non-transfected

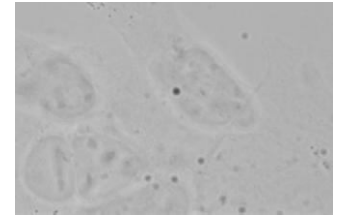
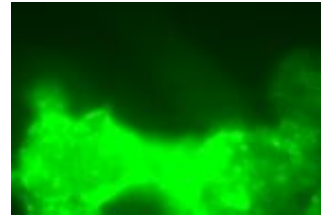
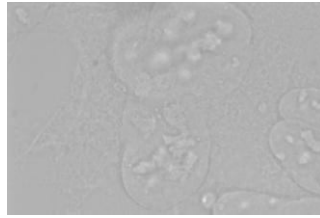
PTH₁R

TSA detection

transmission

TSA detection

transmission

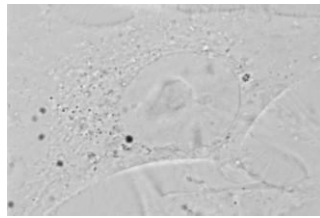


B. MG-63 osteoblastoid cells

control cells

autofluorescence

transmission



25 μm

4A6-AF488 + control CM

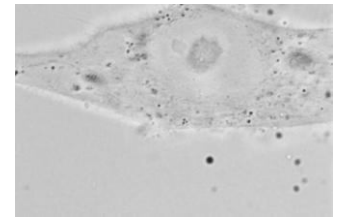
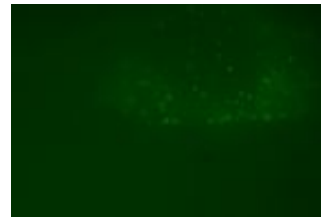
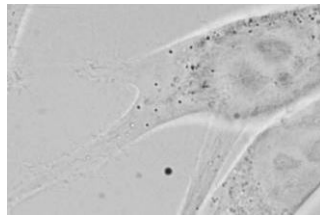
4A6-AF488 + PTH-myc CM

fluorescence

transmission

fluorescence

transmission



4A6 + control CM

4A6 + PTH-myc CM

TSA detection

transmission

TSA detection

transmission

