Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2011 February ; 94(1-2): 53–58. doi:10.1016/j.prostaglandins.2010.12.007.

Evidence for the presence of a critical disulfide bond in the mouse EP3 γ receptor

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Abstract

To determine the contribution of cysteines to the function of the mouse E-prostanoid subtype 3 gamma (mEP3 γ), we tested a series of cysteine-to-alanine mutants. Two of these mutants, C107A and C184A, showed no agonist-dependent activation in a cell-based reporter assay for mEP3 γ , whereas none of the other cysteine-to-alanine mutations disrupted mEP3 γ signal transduction. Total cell membranes prepared from HEK293 cells transfected with mEP3 γ C107A or C184A had no detectable radioligand binding. Other mutant mEP3 γ receptors had radioligand affinities and receptor densities similar to wild-type. Cell-surface ELISA against the N-terminal HA-tag of C107A and C184A demonstrated 40 % and 47 % reductions respectively in receptor protein expression at the cell surface, and no radioligand binding was detected as assessed by intact cell radioligand binding experiments. These data suggest a key role for C107 and C184 in both receptor structure/stability and function and is consistent with the presence of a conserved disulfide bond between C107 and C184 in mouse EP3 that is required for normal receptor expression and function. Our results also indicate that if a second disulfide bond is present in the native receptor it is non-essential for receptor assembly or function.

Keywords

Prostaglandin E₂; mutagenesis; cysteine; disulfide bond; GPCRs*

INTRODUCTION

Prostaglandins are generated by the oxidation of arachidonic acid as catalyzed by the cyclooxygenases and exert their biologic actions through stimulation of a family of prostanoid

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receptors [1,2]. Prostaglandin E₂ (PGE₂) mediates a number of physiologic processes, including fever [3], pain [4], and control of hemodynamics [5,6,7,8], by signaling through four subtypes of differentially expressed E-prostanoid (EP) receptors: EP1, EP2, EP3, and EP4 [9]. Three alternatively spliced C-terminal tails of mouse EP3 receptor, designated mEP3 α , mEP3 β , and mEP3 γ , further add to the diversity of the signaling pathways of PGE₂ [10,11,12,13]; these splice variants have indistinguishable radioligand binding properties. Although the molecular pharmacology of the EP receptors has been studied in detail [14,15,16,17,18], the structural features of the EP receptors themselves have been incompletely characterized.

EP receptors are seven transmembrane domain, Class A G-Protein Coupled Receptors (GPCRs). A number of cysteine residues are highly conserved within GPCRs, suggesting a critical role for these cysteines in receptor function. Evidence suggests a pair of highly conserved cysteines, one in extracellular loop 1 (ECI) or just after that loop at the start of Helix 3 and one in extracellular loop 2 (ECII), which often form a disulfide bond in the extracellular domain of GPCRs [19,20,21,22]. A covalent bond between two such positions is known to constrain helix topology, promote functional tertiary arrangement, and stabilize the ligand-binding pocket of seven transmembrane domain receptors [22,23].

Intracellular cysteine side-chains can be the targets of enzymatic S-acylation and S-alkylation (isoprenylation) reactions. Cysteine residues in the C-terminal tail of GPCRs often have a molecule of palmitic acid covalently attached via a thioester bond [21,22,24] or an isoprene polymer covalently attached via a thioether bond [25,26]. The lipid moieties are thought to insert into the inner leaflet of the plasma membrane, forming a fourth intracellular loop. A number of studies have indicated these post-translational modifications are necessary for proper receptor expression [27,28,29] and function [24,30,31].

Here we evaluate the contribution of each of the 13 cysteines present in mEP3 γ with respect to ligand binding affinity, cell surface expression, and downstream effector coupling of the receptor. While most cysteine-to-alanine mutations were well tolerated, two mutations abrogated detectable radioligand binding and cell signaling and attenuated surface trafficking of the receptor. These cysteines correspond to a pair of conserved cysteines located in ECII and the extracellular end of Helix 3 that are the site of an extracellular disulfide bond in >90% of the Class A GPCRs. Our results indicate that these conserved cysteine residues are important in enabling efficient surface expression and are also required for the function of surface-expressed EP3 receptor.

MATERIALS and METHODS

Materials

HEK293 cells were purchased from ATCC (#CRL-1573, Manassas, VA). PGE₂ and sulprostone were purchased from Cayman Chemical (Ann Arbor, MI). [³H]PGE₂ was purchased from Perkin Elmer (Waltham, MA). Mouse anti-HA mAb, clone 6E2 was purchased from Cell Signaling (Danvers, MA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody was purchased from Jackson ImmunoResearch (West Grove, PA). Indomethacin, sodium butyrate, bovine serum albumin (BSA), and poly-D-lysine were purchased from Sigma Aldrich (St. Louis, MO). Chlorophenolred- β -D-galactopyranoside (CPRG) was purchased from Roche Applied Science (Indianapolis, IN). High-glucose, no L-glutamine Dulbecco's Modified Eagle Medium (DMEM), OptiMEM I, and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). L-glutamine and penicillin/streptomycin were purchased from MediaTech (Manassas, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). HRP substrate kit was purchased from Bio-Rad (Hercules, CA). Bicinchoninic Acid (BCA) Protein Assay kit was purchased from Thermo Scientific (Rockford, IL).

Generation of Mutant HAmEP3 γ cDNA

Mutants of mEP3 γ were generated as previously described [32]. Mutant HAmEP3 γ cDNAs were generated by Mutagenex (Piscataway, NJ) using hemagglutinin (HA)-tagged wild-type mouse EP3 gamma cDNA in pcDNA3 as a template. Wild-type receptor and all mutant receptors contained a single threonine-to-serine variant from published sequence [10] that appears to have no effect on receptor function. Primers used for mutagenesis are listed in Table 1. DNA sequences of mutant receptors were confirmed by Mutagenex and independently at the Vanderbilt DNA Sequencing facility.

Cell Culture

HEK293 cells were maintained at 37 °C / 5% CO₂ in complete media (DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin). Cells were cotransfected with 3 μ g receptor cDNA plasmid and 3 μ g pCRE/lacZ reporter plasmid [33] using Lipofectamine 2000. Six hours after adding DNA-Lipofectamine 2000 complexes, media was replaced with complete media containing 20 μ M indomethacin and 5 mM sodium butyrate. Cells were allowed to recover for 18 to 24 hours before being plated in 96-well plates (5×10^4 cells/well) for the reporter assay and 100 mm dishes to prepare membranes. Cells were incubated for an additional 24 to 48 hours, until confluence was reached.

CRE/LacZ Reporter Assay

HEK293 cells in 96-well plates cotransfected with HAmEP3 γ receptor plasmid and pCRE/lacZ reporter plasmid were incubated with prostaglandin E₂ (1 nM – 1 μ M) in Opti-MEM containing 5 mM sodium butyrate and 20 μ M indomethacin. After cells were stimulated with agonist for 6 hours, media was aspirated and cells were washed with phosphate-buffered saline (PBS). Cells were incubated for 10 minutes at room temperature in 25 μ L of lysis buffer (10 mM sodium phosphate, 0.2 mM MgSO₄, and 10 μ M MnCl₂, pH 8.0). Assay plates were developed as described [34]. Concentration response curves to prostaglandin E₂ were determined by measuring relative enzyme activity as absorbance at 570 nm on Multiskan Ascent plate reader (Thermo Labsystems, Waltham, MA).

Saturation Radioligand Binding

Total cell membranes from HEK293 transfectants used in CRE/LacZ assays described above were prepared as described [12]. Membranes (5 – 10 μ g) were incubated with [³H]PGE₂ (0.25 – 8 nM) in 200 μ L of binding buffer (25 mM potassium phosphate, 1 mM EDTA, and 10 mM MgCl₂, pH 6.2) for 2 hours at 30 °C. Nonspecific binding was determined in the presence of the unlabeled EP1/3 selective agonist sulprostone (5 μ M). Binding reactions were terminated and radioactivity was quantified as previously described [12].

Cell Surface Radioligand Binding

Cells transfected with HAmEP3 γ receptor plasmids were plated 18 hours post-transfection at a density of 2×10^5 cells/well in poly-D-lysine coated 24-well plates in media containing 20 μ M indomethacin and 5 mM sodium butyrate 24 hours prior to assay. Cells were washed with ice-cold 0.5 % FBS in PBS. Cells were incubated with [³H]PGE₂ (5 nM) in the presence and absence of unlabeled sulprostone (5 μ M) for 2 hours on ice. Cells were washed twice with ice-cold PBS and lysed with 0.1 M NaOH. Cell surface radioligand binding was quantified by liquid scintillation counting.

Immunodetection of Cell Surface Receptor Expression

Transfected cells were plated in poly-D-lysine-coated 24-well plates at a density of 2×10^5 cells/well in media containing 20 μ M indomethacin and 5 mM sodium butyrate 24 hours prior to assay. Cells were washed with PBS and fixed with 250 μ L of 3.7 % formalin in Tris-buffered saline (TBS) for 5 minutes at 23 °C and washed with TBS. Cells were blocked with 1 % BSA in TBS for 30 minutes at 23 °C. HA-tagged EP3 receptor was detected by incubation of cells with mouse anti-HA antibody (1:1000 in 1 % BSA) for 1 hour at 23 °C. Cells were washed with TBS and blocked with 1 % BSA in TBS for 15 minutes at 23 °C. HRP-conjugated goat anti-mouse antibody (1:1000 in 1 % BSA) was added to cells for 1 hour at 23 °C. Cells were washed with TBS and developed using HRP substrate kit as prescribed with the exception of using 300 μ L of substrate and stop solutions per well.

RESULTS

To determine the functional importance of each cysteine residue of the mouse EP3 gamma (mEP3 γ) receptor (Figure 1), ligand binding and signaling characteristics were determined for each of 13 cysteine-to-alanine point mutants of HA-tagged wild-type mEP3 γ transiently expressed in HEK293 cells. Signal transduction for each mutant was evaluated by a cell-based CRE reporter assay for mEP3 γ activation [33,34]. As observed previously for the rabbit EP3 receptor, agonist activation of the mouse EP3 receptor lead to a dose dependent increase in CRE reporter activity [34]. Each receptor bearing a cysteine-to-alanine mutation (e.g. C24A) was similarly able to transduce an agonist-stimulated reporter signal, with the exception of receptors with mutations at C107 or C184 (Figure 2).

Radioligand binding affinity (K_D) and receptor density (B_{max}) for wild-type mEP3 γ and each mutant receptor were determined from saturation binding isotherms on broken-cell membranes prepared from transiently transfected HEK293 cells. Radioligand binding was undetectable from two mutant receptors, C107A and C184A, suggesting that if expressed these receptors have K_D values at least 50-fold weaker than wild-type, below the limit of detection (Figure 3). Other receptors had no significant decrease in affinity for [3 H]PGE $_2$ (Table 2). Although as determined by radioligand binding, variation of receptor density in these transient transfectants was statistically significant for many of the mutant receptors, only three of the mutants (C68A, C270A, and C301A) displayed dramatically reduced binding levels, indicative of reduced receptor expression. The lowest receptor B_{max} detected was observed with receptors bearing either C270A or C301A mutations, suggesting that the lower limit of detection in the radioligand-binding assay is at least at this level of protein expression, 0.3 pmol/mg. This is approximately 4 % of the B_{max} observed for wild type receptors (6.9 pmol/mg; Table 2). To evaluate surface expression of nonfunctional mutant receptors, cell surface ELISA was performed. The N-terminal HA-tag epitope of mutants C107A and C184A was detected at the cell surface by ELISA, although at significantly attenuated levels as compared to cells expressing HA-tagged wild-type receptor (Figure 4A). The level of ELISA signal of the mutant receptors was approximately 12 % and 20 % of that observed for the wild-type receptor. We further assessed the ability of the mutant receptors to bind radioligand at the cell surface in intact cells. Cell surface radioligand binding was undetectable however, suggesting that the population of C107A and C184A receptors that were trafficked to the cell surface was unable to bind ligand (Figure 4B).

DISCUSSION

To facilitate structural studies of integral membrane proteins, our lab and others [35,36,37] have utilized mutant proteins lacking free cysteine residues; a protein lacking the free sulfhydryl groups of cysteine residues are less prone to irreversible aggregation and can be used for site-directed chemical modifications. In this study we characterized the phenotype of

individual cysteine-to-alanine mutations of each cysteine residue in the mouse EP3 γ receptor. We found that substitutions at 11 of the 13 cysteine residues in mEP3 γ were well tolerated with respect to receptor expression, ligand affinity, and signal transduction as assessed by radioligand binding and cell-based signal transduction assays.

Two cysteine residues at sites that correspond to an extracellular disulfide bond highly conserved among Class A GPCRs were required both for efficient cell surface expression and for the function of that population of the receptor that reaches the cell surface. If the disulfide bond is required and these cysteines do indeed compose a disulfide crosslink, loss of function and efficient trafficking should result from mutating either cysteine of the pair as was observed for C107 and C184 of mEP3 γ . It is interesting to note that previous studies on the rabbit ortholog of the mouse EP3 receptor (EP3 77A) showed no dependency for the cysteine residue on the extracellular end of Helix 3 (corresponding to C184 in mEP3 γ) [17]. The region of ECII distal to C184 is one of least conserved regions of the entire receptor between mouse and rabbit sequences; this may contribute in part to the interspecific functional differences in the EP3 receptor.

Data presented here show multiple defects in receptors having disulfide bonded cysteines mutated. Cell surface ELISA (Figure 4B) demonstrated that C107A and C184A mutant receptors trafficked to the cell surface in significantly reduced numbers. The highly conserved disulfide bond is likely required to achieve and maintain proper tertiary structure and stability of a GPCR [38]; misfolded receptors may be retained within the cell [39].

The reduced trafficking seen for the C107A and C184A mutant forms of mEP3 γ may be due to much of the newly translated receptor failing to fold properly in the endoplasmic reticulum and being targeted for degradation by ER protein folding quality control. Based on experiments with model membrane proteins and cell biological characterization of ER quality control, it has been argued that most mutations that lead to targeting of nascent membrane proteins for degradation are mutations that result in thermodynamic destabilization of those proteins [40]. It is therefore probable the stability of C107A and C184A receptors is decreased with respect to the wild-type receptor.

While C107A and C184A mutants have trafficking defects, the remaining receptor that does traffic to the cell surface appears to be nonfunctional. These mutants have detectable cell surface expression as demonstrated by ELISA at 12 and 20% of wild-type levels respectively (Figure 4A); neither displayed detectable radioligand binding in assays of intact cells (Figure 4B) or isolated membranes (Figure 3). In contrast, we could detect ligand binding down to at least 4% of wild type levels for C270A and C301A, suggesting that the lack of binding observed for C107A and C184A is not due to insufficient sensitivity of the radioligand-binding assay (Table 2). Similarly, neither mutant exhibited receptor activation in the CRE reporter assay (Figure 2) although C270A and C310A displayed robust signaling despite their reduced expression levels. The loss of function phenotype observed for C107A and C184A is consistent with data from other investigators showing a role for GPCR disulfide bonds in ligand binding and receptor-effector coupling [41,42,43].

Intracellular cysteine residues of GPCRs may be targets of lipid modification. Cysteine residues in the intracellular C-terminal tail of GPCRs may be palmitoylated or, less frequently, isoprenylated. These lipid modifications can be required for receptor expression [27,28,29] and function [24,30,31]. However, if mEP3 γ is lipid modified, the modification does not appear to be necessary for receptor expression or function. This has also been observed for other GPCRs including the α_{2A} adrenergic receptor (α_{2A} AR), which is palmitoylated, but mutation of the palmitoylation site does not affect ligand binding or effector coupling to α_{2A} AR [44], consistent with the idea that receptor palmitoylation is not required for receptor function.

In the transmembrane environment of the seven-helix bundle, cysteine side chains can participate in hydrogen bonding networks [45,46]. Hwa and colleagues reported deficits in expression of the human prostacyclin receptor when any of three conserved, transmembrane cysteines were mutated to alanines [42]. Similarly, as noted above, attenuated receptor expression, as evidenced by greater than ten-fold lower $B_{\max}$ than wild-type mEP3 γ was observed for C270A, C301A as well as C68A. Cysteine residues 68 and 301 are conserved across orthologs of EP3. Cysteine 270 is conserved across the entire superfamily of GPCRs [17]. While the variance in $B_{\max}$ values may be an artifact of a transiently-transfected system, the reduction in expression of these mutant receptors while maintaining high affinity for [3 H] PGE $_2$ could suggest a role for these residues in maintaining the stability of the receptor, potentially by hydrogen bonding interactions. Further experiments are needed to evaluate this hypothesis.

In summary, we have shown through mutagenesis that 11 of 13 of cysteine residues present in mEP3 γ are not required for receptor trafficking, ligand binding, or signal transduction. One pair of cysteine residues that likely comprise a disulfide bond between ECI and ECII is required for proper function and cell surface expression of the receptor. While future experiments are required to confirm the precise nature of the defect caused by these mutations, these data begin to cast light on some of the structural characteristics of EP receptors.

ABBREVIATIONS

mEP3 γ	mouse E-prostanoid receptor subtype 3 gamma
PGE $_2$	prostaglandin E2
HA	hemagglutinin epitope tag

Acknowledgments

This work was supported in part by National Institutes of Health grants R01 DK037097, R01 DK046205, P50 GM15431, Roadmap Grant R01 GM081816, and Integrative Training in Therapeutic Discovery Training Program T90 DA022873.

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Figure 2. CRE/LacZ cell signaling assay. HEK293 cells transiently coexpressing the pCRE/LacZ reporter plasmid with either wild-type HA-mEP3 γ cDNA, mutant HA-mEP3 γ cDNA, or empty pcDNA3 vector. A) Concentration response curves of (Δ) wild-type, ($\square$) C24A, and (∇) C68A transfected and ($\diamond$) pcDNA3 transfected cells to PGE₂. B) Concentration response curves of ($\times$) C107A, ($\square$) C184A, (∇) C212A, and ($\circ$) C224A transfected cells to PGE₂. C) Concentration response curves of ($\square$) C236A, (∇) C265A, and ($\circ$) C270A transfected cells to PGE₂. D) Concentration response curves of ($\square$) C291A, (∇) C301A, ($\circ$) C334A, and ($\times$) C343A transfected cells to PGE₂. Wild-type and pcDNA3 transfectant concentration response curves from A were replotted in each panel for comparison. Data are means $\pm$ SEM of between three and eight independent experiments performed in triplicate and normalized to wild-type receptor and background reporter signal.

Figure 3.

Saturation radioligand binding curves for membranes prepared from (■) wild-type HA-mEP3γ, mutant HA-mEP3γ, and pcDNA3 transiently-transfected HEK293 cells. Specific binding of 5 nM [³H]PGE₂ was undetectable for (○) pcDNA3, (□) C107A, and (△) C184A samples; these symbols are manually displaced horizontally for clarity. Shown are representative data (mean ± SEM) from three separate experiments conducted in triplicate.

Figure 4.

Surface expression of wild-type and mutant HA-mEP3 γ receptors. A) Cell Surface ELISA. Anti-HA detection of N-terminus receptor located in extracellular domain of cell detected by horseradish peroxidase activity and expressed as absorbance at 415 nm (mean $\pm$ SEM). Shown are representative data from three separate experiments conducted in triplicate. * $P < 0.001$ vs. WT, † $P < 0.05$ vs. pcDNA3. B) Intact cell radioligand binding. Binding of [³H]PGE₂ to the surface of intact HEK293 cells expressing wild-type and mutant HA-mEP3 γ receptors expressed as liquid scintillation counts per minute (mean $\pm$ SEM). Shown are representative data from three separate experiments conducted in triplicate. * $P < 0.001$.

Table 1
Primers used in mutagenesis

Forward and reverse primers used by Mutagenex to produce missense mutations in wild-type mEP3 γ plasmid. Lowercase nucleotides indicate the mutagenic sequence.

Mutant	Primer ID	Primer Sequence
C24A	403-1F	5 -AAAGGTCTCGgctGGCTCCGTGTCCGTGG
	403-1R	5 -AAAGGTCTCCCAGCGTCGTCGGTAGTACTT
C68A	403-2F	5 -AAAGGTCTCGgctATTGGCTGGCTGGCGC
	403-2R	5 -AAAGGTCTCATAGCCAGCAGGAAAGACTTCTT
C107A	403-3F	5 -AAAGGTCTCGgctACCTTCTTCGGGCTAAC
	403-3R	5 -AAAGGTCTCGTAGCCAGACGCCCCGATGG
C184A	403-4F	5 -AAAGGTCTCgctTTCATCAGCACCGGGCCG
	403-4R	5 -AAAGGTCTCGAAAGCCCACGTGCCCCGGCCA
C212A	403-5F	5 -AAAGGTCTCgctTTGGGCTTGCTGGCTCTG
	403-5R	5 -AAAGGTCTCCAAGCGGCGAAGGCGGAGG
C224A	403-6F	5 -AAAGGTCTCCgctAACCTGGCGACCATCAA
	403-6R	5 -AAAGGTCTCTTAGCGGCAAAGGTCACCACCA
C236A	403-7F	5 -AAAGGTCTCGCgctCGGGCCAAAGCCGC
	403-7R	5 -AAAGGTCTCGAGCGCGGGACACCAGGGCT
C265A	403-8F	5 -AAAGGTCTCGgctTGTGCTGTCCGTCTGTTG
	403-8R	5 -AAAGGTCTCACAGCCATGATCCCCATGAGCT
C270A	403-9F	5 -AAAGGTCTCTCgctTTGGTCGCCGCTATTGA
	403-9R	5 -AAAGGTCTCAAGCGACGGACAGCACACACA
C291A	403-10F	5 -AAAGGTCTCAgctAAGACACAGATGGGAAAG
	403-10R	5 -AAAGGTCTCTTAGCTTGCTCAACCGACATCTG
C301A	403-11F	5 -AAAGGTCTCGgctAATTCCTTTCTAATTGCAGT
	403-11R	5 -AAAGGTCTCTTAGCCTCCTTCTCCTTTCCCAT
C334A	403-12F	5 -AAAGGTCTCTCgctCAGGTAGCAAACGCTGT
	403-12R	5 -AAAGGTCTCGAGCGAACTTCCGAAGAAGGAT
C343A	403-13F	5 -AAAGGTCTCgctTCTAGTGATGGACAGAAAG
	403-13R	5 -AAAGGTCTCAGAAGCACTGGAGACAGCGTTTG

Table 2
Phenotypic analysis of cysteine mutants

Summarized results from saturation binding isotherm experiments (mean $\pm$ SEM) collected for wild-type and mutant HAmEP3 γ .

Genotype	K _D (nM)	B _{max} (pmol/mg)
Wild-Type	2.6 $\pm$ 0.4 (3)	6.9 $\pm$ 0.4 (3)
C24A	1.2 $\pm$ 0.3 (3) ^a	5.0 $\pm$ 0.4 (3) ^a
C68A	0.7 $\pm$ 0.3 (4) ^a	0.4 $\pm$ 0.1 (4) ^c
C107A	N.D. (3)	N.D. (3)
C184A	N.D. (3)	N.D. (3)
C212A	2.1 $\pm$ 0.7 (3)	1.6 $\pm$ 0.5 (3) ^b
C224A	1.3 $\pm$ 0.5 (3)	1.6 $\pm$ 0.7 (3) ^a
C236A	1.7 $\pm$ 0.6 (3)	2.4 $\pm$ 1.0 (3) ^a
C265A	2.0 $\pm$ 0.5 (3)	1.2 $\pm$ 0.1 (3) ^b
C270A	0.7 $\pm$ 0.1 (3) ^a	0.3 $\pm$ 0.1 (4) ^c
C291A	1.9 $\pm$ 0.6 (3)	1.2 $\pm$ 0.2 (3) ^b
C301A	0.6 $\pm$ 0.1 (3) ^a	0.3 $\pm$ 0.03 (3) ^c
C334A	1.3 $\pm$ 0.4 (3) ^a	1.6 $\pm$ 0.7 (3) ^a
C343A	2.0 $\pm$ 0.5 (3)	2.7 $\pm$ 1.3 (3) ^a

^aP < 0.05,

^bP < 0.001,

^cP < 0.0001 by two-tailed t-test vs. wild-type.

N.D. not detected.