AWARD NUMBER: W81XWH-12-1-0213

TITLE: Targeting G-Protein Signaling for the Therapeutics of Prostate Tumor Bone Metastases and the Associated Chronic Bone Pain

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REPORT DATE: September 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2015	Final	1 July 2012 - 30 June 2015

4. TITLE AND SUBTITLE
Targeting G-Protein Signaling for the Therapeutics of Prostate Tumor Bone Metastases and the Associated Chronic Bone Pain

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0213

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Iowa
Iowa City, IA 52242

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Bone tumor metastasis is the major cause of mortality and morbidity in advanced prostate cancer patients. These patients frequently suffer from moderate to severe chronic bone pain. However, the current treatments for these patients are ineffective and non-curative, because metastatic tumors are resistant to most of the current anti-cancer treatments, and the currently used pain therapeutic medications or analgesics often do not provide effective relief from pain due to the development of tolerance upon chronic use, and also have serious side effects. Thus, it is imperative to develop novel approaches that can both effectively block tumor growth in bones and relieve the associated bone cancer pain. This proposal aims to define the key role of the Gβγ subunits of heterotrimeric G proteins in the development of prostate tumor bone metastasis and the associated bone pain. Our studies have demonstrated that Gβγ signaling plays a key role in mediating proliferation and migration of several prostate cancer cell lines in vitro, including PC3, DU145 and 22Rv1. Importantly, inhibition of Gβγ signaling did not affect the proliferation of non-transformed prostate epithelia cells RWPE-1, suggesting that Gβγ signaling may be specifically required for malignant cell growth. Moreover, we showed that inducible inhibition of Gβγ signaling in 22RV1 cells blocked the growth of pre-established primary tumor growth in the prostate, the formation of metastases in multiple organs (including bone metastases in the limbs) of nude mice, and prolonged mouse survival. Notably, the reduction of bone metastases was so dramatic (5 out of 7 mice injected with control cells versus 1 out of 7 mice injected with Gβγ signaling-deficient cells developed bone metastases) such that the assessment of the bone metastasis-associated bone pain in these mice became impractical. Blocking Gβγ signaling also enhanced the therapeutic efficacy of paclitaxel in the PC3 xenografts. Additional studies indicate that Gβγ signaling may stimulate prostate cancer cell growth and migration through several downstream signaling pathways, including PI3K/AKT and ERK. Together, our results provide compelling evidence for the involvement of Gβγ signaling in prostate cancer progression and support for targeting Gβγ as a novel therapeutic approach for prostate cancer.

15. SUBJECT TERMS
Cancer Pain, Prostate Cancer Bone Metastasis, heterotrimeric G protein βγ subunits, G protein-coupled receptors, signal transduction

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	11	

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1. Introduction:

Bone metastasis is one of the most common and severe complications in advanced prostate cancer. It is the major cause of mortality and morbidities, due to the development of bone pain, hypercalcemia, fractures, spinal cord compression and consequent paralysis. The current regimen for these patients is largely palliative and non-curative, because metastatic tumors are resistant to most of the current anti-cancer treatments. Thus, it is imperative to develop novel therapeutic approaches for the treatment of advance prostate cancer. This proposal aims to define the key role of the Gβγ subunits of heterotrimeric G proteins in the development of prostate tumor bone metastasis and the associated bone pain, as well as determine the potential therapeutic efficacy of targeting Gβγ with small molecule inhibitors in preclinical models of bone-metastasized prostate cancer. G proteins mediate the function of a large group of cell surface receptor proteins called G protein-coupled receptors (GPCRs). Comparative experimental and clinical evidence has indicated that excessive activation of the GPCR systems due to overexpression of the receptors and their ligands in prostate tumor cells or their surrounding cells contributes to the metastatic spread of tumor cells to bones, their subsequent growth there and the consequent bone destruction. Moreover, continue activation of GPCRs in the sensory nerve fibers adjacent to bones results in increased activity/expression of key pain-sensing receptor channels, such as TRPV1, such that the channels are constitutively activated, leading to the sensation of chronic pain without any overt stimulation. Thus, the GPCR system represents an attractive target for the therapeutics of bone tumor metastasis and the associated bone pain. However, the involvement of several dozen GPCRs and their ligands in tumor progress has presented a significant hurdle for the progression of such approach. Given that G proteins function downstream of GPCRs, we propose to thoroughly investigate the role of Gβγ in mediating signals from multiple GPCRs to promote prostate tumor growth and metastasis and for the associated bone cancer pain, using both *in vitro* cell culture and *in vivo* preclinical model of prostate tumor metastasis. Considering the recent discovery of a series of small molecule inhibitors of Gβγ that have been successfully used in the treatment of several pathologies in the preclinical mouse models of heart failure, inflammation, opioid receptor-dependent analgesia and morphine-induced antinociceptive tolerance and dependence, without causing overt side effects, results from our proposed studies have the potential to uncover a novel and efficacious approach for the development of new mechanism-based therapies to improve the outcome of advanced prostate cancer patients, including the men in the military services who are suffering from this disease.

2. Keywords: Prostate Cancer Bone Metastasis, Bone Cancer Pain, Heterotrimeric G protein betagamma subunits, G protein coupled receptors (GPCRs), TRPV1, Nociceptor Sensitization

3. Overall project summary: Summarized below are the accomplishments from research work in direct alignment to task outlined in the Statement of Work (SOW).

Milestone-1: Determine the role of Gβγ signaling in mediating prostate tumor cell growth, migration and invasion *in vitro*, as well as mediating GPCR-regulated TRPV1 channel function in cultured mouse sensory neurons (Aim 1)

Major Goal/Objective 1: Determine the role of Gβγ signaling in regulating prostate tumor cell growth, migration and invasion.

Accomplishments: To determine the function of Gβγ signaling in prostate cancer cells, we use lentiviruses to generate stable cell lines expressing tetracycline-inducible GFP as control or Gαt, a scavenger of Gβγ, at a comparable level in the castration-resistant prostate cancer cell lines, PC3, DU145, 22RV1, and an untransformed prostate epithelial cell line, RWPE1 (Fig. 1A). To assess the specific effect of Gαt expression on Gβγ signaling, we determined AKT phosphorylation stimulated by several GPCR agonists, including LPA and SDF1α that are known to activate AKT via Gβγ-mediated PI3Kβ. As control, we also stimulated cells with IGF or EGF, which activates AKT via receptor tyrosine kinases. As shown in Fig. 1B-D, Gαt expression selectively inhibited AKT phosphorylation induced by GPCR agonists but not IGF or EGF, in all cell lines examined. LPA-stimulated AKT phosphorylation was also sensitive to pertussis toxin treatment, suggesting the involvement of Gβγ released from Gi/o-coupled GPCRs. Together, our data indicate that Gαt expression selectively abolished G signaling from multiple GPCRs in prostate cancer cells.

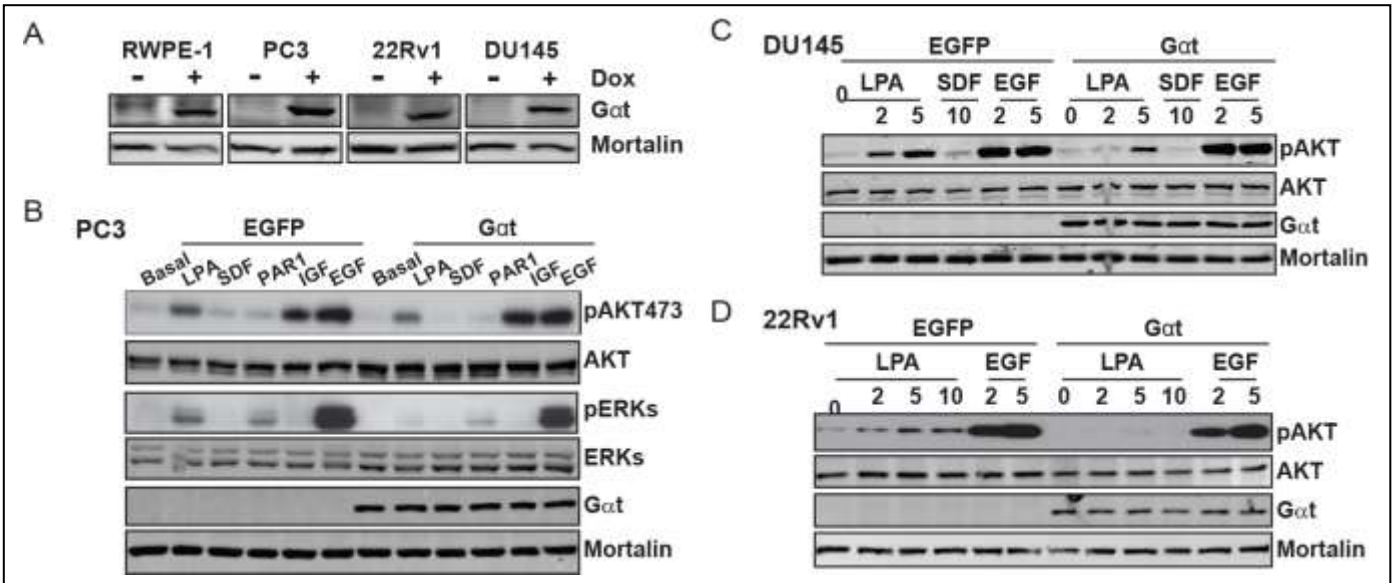

Fig 1. Induced Gαt expression selectively blocks GPCR-mediated signaling in prostate cancer cells. A, Induced Gαt expression in RWPE-1, PC3, 22Rv1 and DU145 cell lines transduced with lentivirus encoding Gαt and treated with doxycycline (1μg/ml) for three days. **B-D,** Induced Gαt expression inhibited GPCR-stimulated signal transduction in prostate cancer cell lines. Cells were treated with doxycycline to induce GFP or Gαt expression for 3-5 days and then stimulated with LPA (10 μM), SDF1α (50 nM), PAR1 agonist peptide (20 μM), IGF (100ng/ml) or EGF (20 ng/ml) for 5 min or the indicated times. Representative images from at least three independent experiments are shown.

We then investigated the effect of blocking Gβγ signaling on cell growth. As compared to GFP expression, Gαt expression significantly decreased PC3, DU145 and 22Rv1 cell growth in two-dimensional culture in a XTT-based cell viability assay and a 5-bromo-2'-deoxyuridine (Brdu) incorporation assay (Fig. 2A-B). The decreased growth of prostate cancer cells expressing Gαt was also shown by a 50-80% reduction in the size of cell colonies in Matrigel (Fig. 2D). Treatment of prostate cancer cells with PTx reduced the growth of GFP-expressing cells to a level comparable to that of Gαt-expressing cells (Fig.2A), indicating the involvement of Gβγ subunits released from Gi/o proteins. Interestingly, the growth of RWPE1 cells was neither affected by PTx treatment nor Gαt expression (Fig. 2A). These findings suggest that Gβγ signaling is critical for the proliferation of prostate cancer cells but is dispensable for the growth of normal prostate epithelial cells.

Next, we evaluated the role of Gβγ signaling in prostate cancer cell migration. Gαt expression blocked transwell migration of PC3, DU145 and 22Rv1 cells towards individual GPCR agonists, LPA, SDF1α, and a PAR1 peptide agonist, but had no effect on EGF-induced cell migration (Fig. 3A-C). The impaired PC3 cell migration by Gαt expression was also shown in a wound-healing assay (data not shown).

Major Goal/Objective 2: Determine the role of Gβγ signaling in mediating GPCR-stimulated TRPV1 expression/function in cultured mouse DRG sensory neurons.

Accomplishments: we initially attempted to manipulate Gβγ signaling in isolated DRG neurons by overexpressing Gβ1γ2 or Gαt using adenovirus. However, we found that transduction of cells with control adenovirus alone caused a dramatic increase in AKT activation. Since AKT activation will activate TRPV1 channel in DRG neurons, we cannot further assess the effect of Gβ1γ2 and Gαt expression on TRPV1 activity. We have explored the possibility of overexpressing Gβ1γ2 and Gαt in DRG neurons using other approaches including lentivirus- and associate adenovirus-mediated transduction. Unfortunately, all other approaches could not transduce the DRG neurons with high efficiency. Because of this problem, we could not accomplish the objective 2.

Milestone-2: Determine the efficacy of blocking Gβγ signaling on prostate tumor bone metastasis, and chronic bone pain in a xenograft mouse model (Aim 2).

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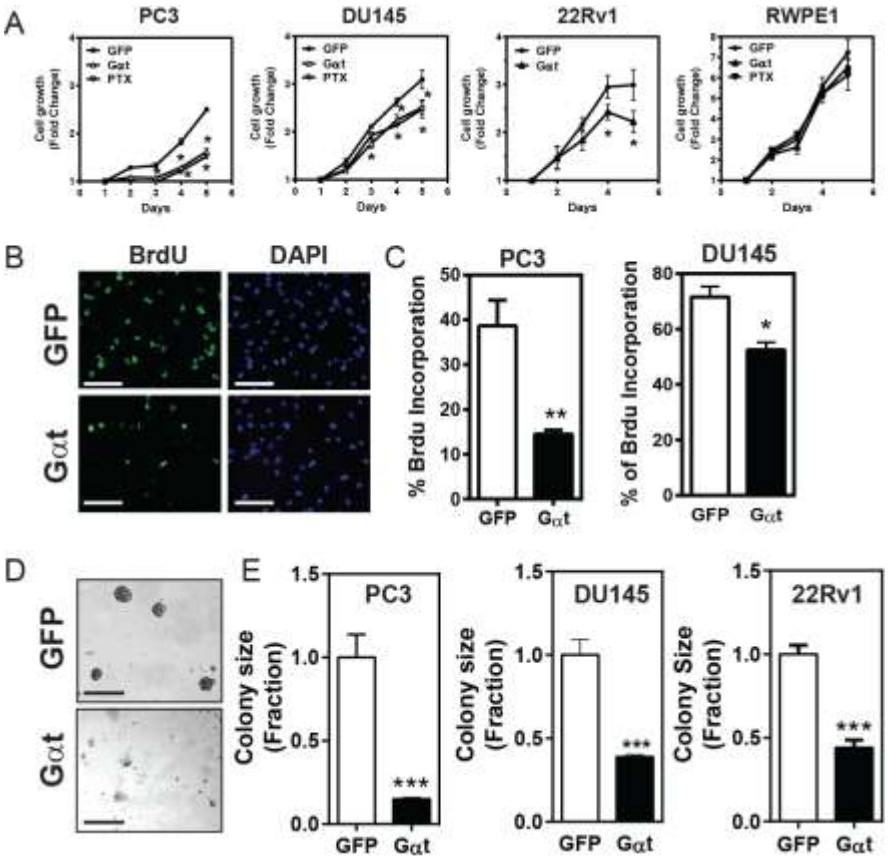

Fig 2. Gαt expression decreases prostate cancer cell proliferation. The indicated cells were treated with doxycyline (1 μg/ml) to induce GFP or Gαt expression. In some cases (A), GFP-expressing cells (PC3, DU145 and RWPE1) were also treated with pertussis toxin (PTx; 0.2 μg). A-C, the effect on cell growth in 2D culture was measured by XTT assays (A) or BrdU incorporation assays (B-C). BrdU incorporation was detected by immunofluorescence staining. Representative fluorescence images of BrdU and nuclear (DAPI) staining in PC3 cells are shown in B. Quantitative data showing the level of BrdU incorporation in PC3 and DU145 are expressed as the percentage of cells stained with BrdU (C). *, ** p<0.05 and 0.01 *versus* GFP, respectively (n=3-4). D-E, the effect on cell growth in Matrigel was determined by phase-contrast imaging, followed by quantification of the size of the colonies. Colony size is expressed as the fraction of that derived from GFP-expressing cells. Representative images of GFP- and Gαt-expressing PC3 cells grown in Matrigel are shown in D. Scale, 100 μm. *** p<0.001 *versus* GFP (n=3-5)

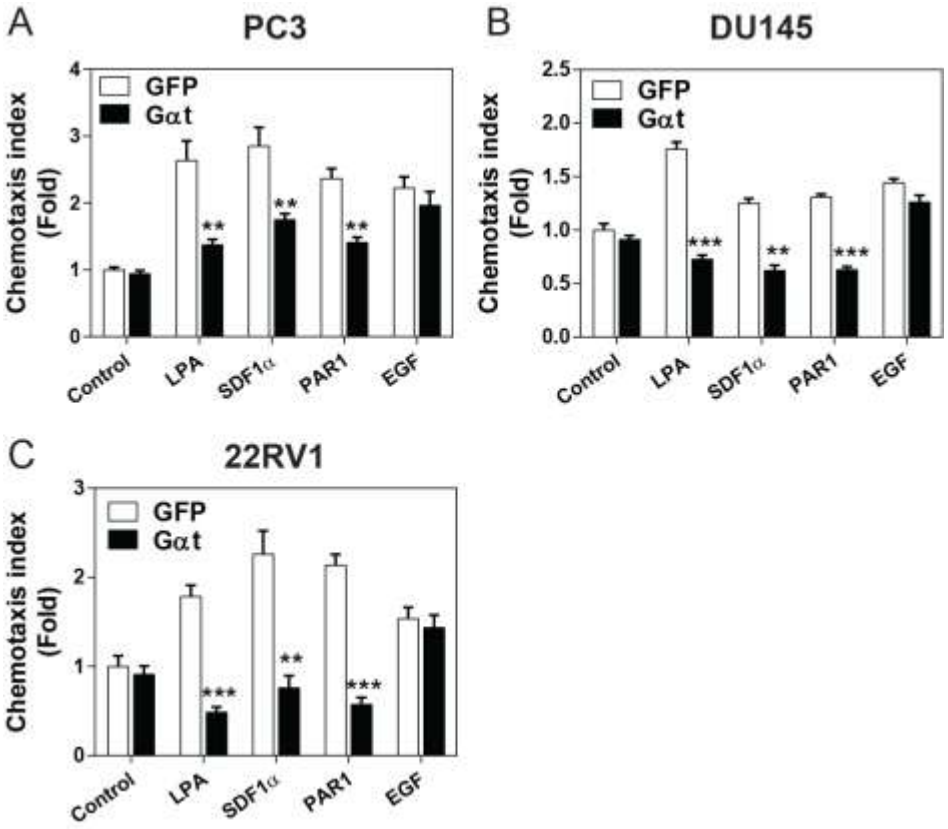

Fig 3. Induced Gαt expression impedes GPCR-induced prostate cancer cell migration. GFP or Gαt was induced by doxycycline for 5 days in PC3 (A), DU145 (B) and 22Rv1 (C). The effects on cell migration were by a transwell migration assay in response to buffer (control) or chemoattractants, LPA (10 nM), SDF1α (100 nM), PAR1 agonist peptide (10 μM) and EGF (50 ng/ml). **, *** p<0.01 and 0.001, respectively, *versus* GFP (n=3-4).

Major Goal/Objective 1: Development of *scid* mouse xenografts of human prostate cancer cells and characterization/assessment of the effects of blocking Gβγ signaling on the formation of bone tumor metastases and bone-related pain behavior in these mice.

Accomplishments: To examine whether blocking Gβγ signaling affects prostate cancer cell growth *in vivo*, we inoculated equal number of 22Rv1 cells expressing inducible GFP or Gαt into the prostate gland of nude mice. In the absence of doxycycline-induced GFP and Gαt expression, the growth rate of Gαt-expressing cells was slightly higher than GFP-expressing cells (Fig. 4A-B). At day 23 post inoculation, BLI revealed an approximately 2-fold higher number of Gαt-expressing cells than GFP-expressing cells, probably due to more Gαt-expressing cells initially inoculated into the prostate gland (Fig. 4A-B). However, upon doxycycline-induced GFP and Gαt expression, the growth of Gαt-expressing cells was almost completely blocked, while the number of GFP-expressing cells was increased for nearly 800-fold after 57 days of inoculation (Fig. 4A-B). These findings indicate that Gβγ signaling is critical for the progression of established 22Rv1 tumor xenograft.

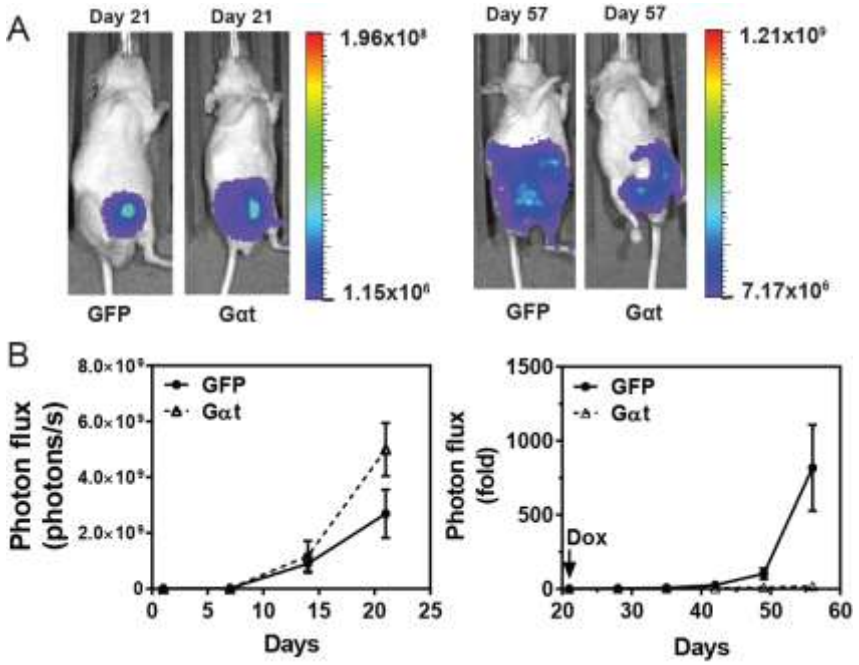

Fig 4. Induced Gαt expression blocks primary prostate cancer growth. 22Rv1 expressing luciferase and inducible GFP or Gαt were implanted into the prostate of nude mice (n=6). 21 days after implantation, mice were fed with doxycycline-containing diets to induce the expression of GFP and Gαt. Tumor growth was monitored by bioluminescence imaging. Representative bioluminescence images (A) and quantitative data (B) showing primary tumor growth at the indicated times.

To determine if Gβγ signaling also promotes prostate cancer metastasis, we injected 22Rv1 cells expressing inducible GFP or Gαt into the left ventricle of nude mice to disseminate tumor cells to multiple organs, and then allowed the cells to form tumors in the absence of doxycycline-induced GFP or Gαt expression for 23 days. Over this period, the growth rate of cells in the whole body was comparable, as revealed by BLI (Fig. 5C). Upon doxycycline-induced GFP or Gαt expression, the growth rate of Gαt-expressing cells in the whole body was slower than GFP-expressing cells but did not reached scientifically significance (Fig. 5D). However, ex vivo BLI revealed that the frequency of tumor formation in multiple organs (i.e., brain, lung, liver, kidney, spleen, leg and mandible) from Gαt-expressing cells was lower than GFP-expressing cells (Table 1). Moreover, mice bearing Gαt-expressing cells showed a significant improvement in overall survival (Fig. 5E). Similar results were found for PC3 cells (Fig. 5F and table 2). These findings indicate that G signaling is also critical for the formation of prostate cancer metastases.

Because of the dramatic reduction (32% and 71% for control 22Rv1 and PC3 cells, respectively *versus* 17% and 14% for Gαt-experssing 22Rv1 and PC3 cells, respectively; table 1 and 2) in bone tumor formation in the limbs of nude mice with 22Rv1-Gαt and PC3-Gαt xenografts, it becomes impossible to generate sufficient number of mice with bone metastases for assessing bone-related pain behavior. Because of this, the effect of blocking Gβγ signaling on bone-related pain behavior in these mice could not be further pursued.

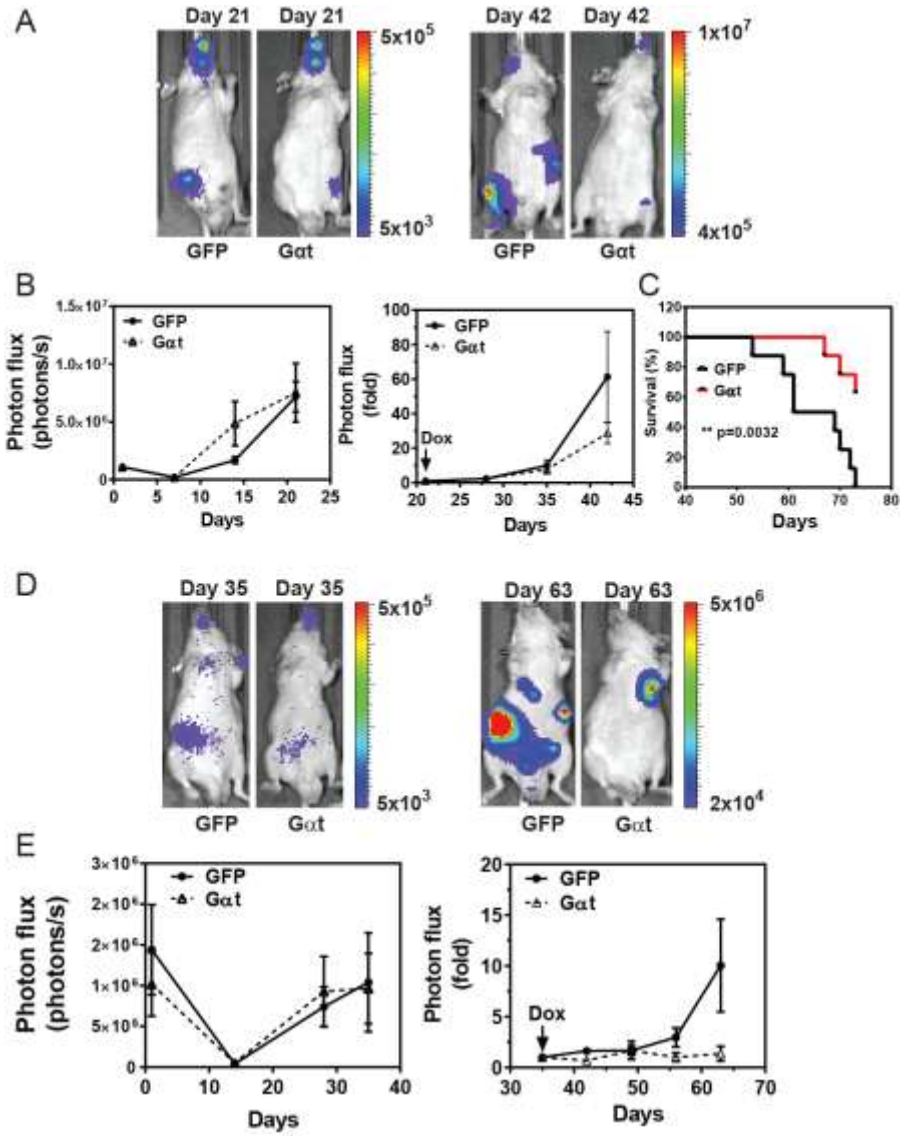

Fig 5. Induced Gαt expression reduces prostate cancer metastasis and increased survival. Nude mice (n=6-7) were inoculated with 22Rv1 (A-C) or PC3 (D-E) cells by intracardiac injection. 21 (A-C) or 35 (D-E) post injection, mice were fed with doxycycline-containing diets to induce GFP and Gαt expression. Tumor growth was monitored by bioluminescence imaging. Representative bioluminescence images (A and D) and quantitative data (B and E) showing primary tumor growth at the indicated times. C, overall survival curve of mice inoculated with 22Rv1 cells.

Table 1. The frequency of PC3 tumor metastasis formation at various tissues of nude mice inoculated with PC3 cells expressing inducible GFP or Gαt via intracardiac injection. The number and percentage of mice detected with tumors at the indicated tissues by *ex vivo* BLI are indicated.

Tissues	GFP (n=7)	Gαt (n=7)
Brain	2 (27.5%)	0 (0%)
Fore Leg-Left	1 (14.2%)	0 (0%)
Fore Leg-Right	1 (14.2%)	0 (0%)
Hind Leg-Left	2 (28.5%)	1 (14.2%
Hind Leg-Right	1 (14.2%)	0 (0%)
Liver	2 (28.5%)	0 (0%)
Lung	3 (42.8)	1 (14.2%)
Kidney	2 (28.5%)	1 (14.2%
Mandible	3 (42.8%)	3 (42.8%)
Spleen	2 (28.5%)	0 (0%)

Table 2. The frequency of 22Rv1 tumor metastasis formation at various tissues of nude mice inoculated with PC3 cells expressing inducible GFP or Gαt via intracardiac injection. The number and percentage of mice detected with tumors at the indicated tissues by *ex vivo* BLI are indicated.

Tissues	GFP (n=6)	Gαt (n=6)
Brain	3 (50%)	1 (16.6%)
Fore Leg-Right	1 (16.6%)	0
Hind Leg-Left	1 (16.6%)	0
Hind Leg-Right	0	1(16.6%)
Lung	1 (16.6%)	0
Kidney	1 (16.6%)	0
Mandible	5 (83.3%)	4 (66.6%)

Major Goal/Objective 2: Determine the role of TRPV1 in chronic pain associated with bone metastasis in nude mice with xenografts for prostate cancer cells.

Accomplishments: this aim was not pursued as explained above.

Major Goal/Objective 3: Assessing the efficacy of blocking Gβγ signaling by systemic delivery of a Gβγ inhibitor.

Accomplishments: We found that subcutaneous implantation of PC3 cells grown under tumorsphere-forming conditions into nude mice generated tumors in a dose-dependent manner (Table 3). Blocking Gβγ signaling in PC3 cells by induced Gαt expression significantly reduced the tumor-forming capacity of PC3 cells, an effect was reproduced by systemic delivery of the Gβγ inhibitor galleon (Table 3). However, the effect of gallein was less than Gαt, probably due to incomplete inhibition of Gβγ signaling at the dose used. Immunohistochemical analysis revealed that blocking Gβγ signaling by Gαt expression or gallein treatment significantly reduced prostate cancer cell proliferation and increased apoptosis in the xenograft tumors, as detected by Ki67 and cleaved caspase 3 staining (Fig. 6).

Notably, inhibiting Gβγ signaling also enhanced the therapeutic efficacy of paclitaxel, a chemotherapeutic reagent used for treating advanced prostate cancer patients (Fig. 7). Together, these findings provide a proof-of-concept for targeting Gβγ as a potential novel therapeutic approach in prostate cancer.

Table 3. The frequency of tumor formation in nude mice (n=5) inoculated with the indicated number of single PC3 cells dissociated from the third passages of tumorspheres grown in ultralow-adhesive plates. Immediately post inoculation of PC3 cells, mice were fed with doxycycline-containing diet to induce GFP or Gαt expression. One group of mice inoculated with GFP-expressing PC3 cells was also treated with gallein (10 mg/kg, twice daily). Mice were monitored weekly for palpable tumor for two months.

Number of cells	50, 000	20, 000	10, 000
GFP	100 %	60 %	80 %
Gαt	100 %	40 %	0 %
GFP+Gallein	100 %	80 %	20 %

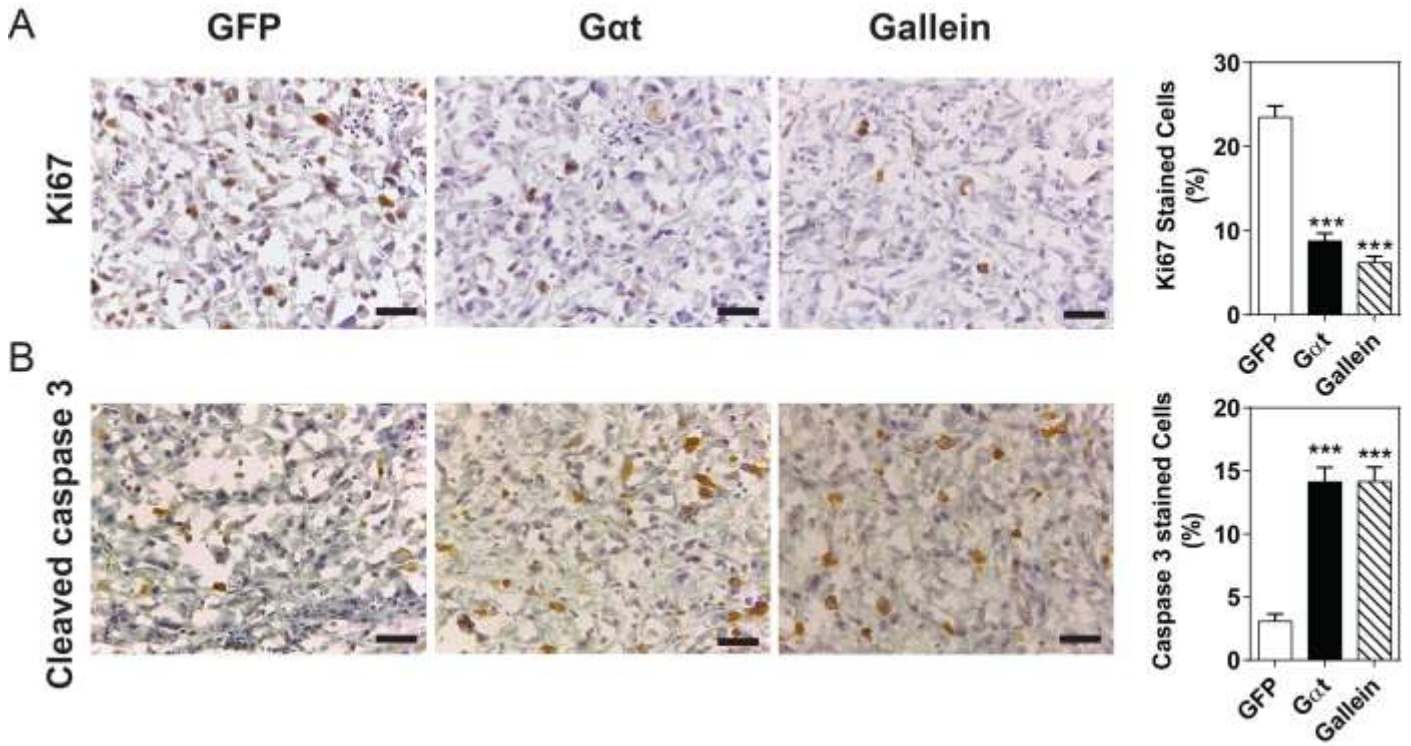

Fig 6. Blocking Gβγ signaling reduces proliferation and increases apoptosis of prostate cancer cells . Tumor sections were prepared from mice inoculated with 10, 000 PC3 cells as indicated in table 3. Sections were stained with Ki67 (A) or cleaved caspase 3 (B). Representative images of the indicated tumor sections and quantitative data are shown. *** p<0.001 *vs* GFP, n=5.

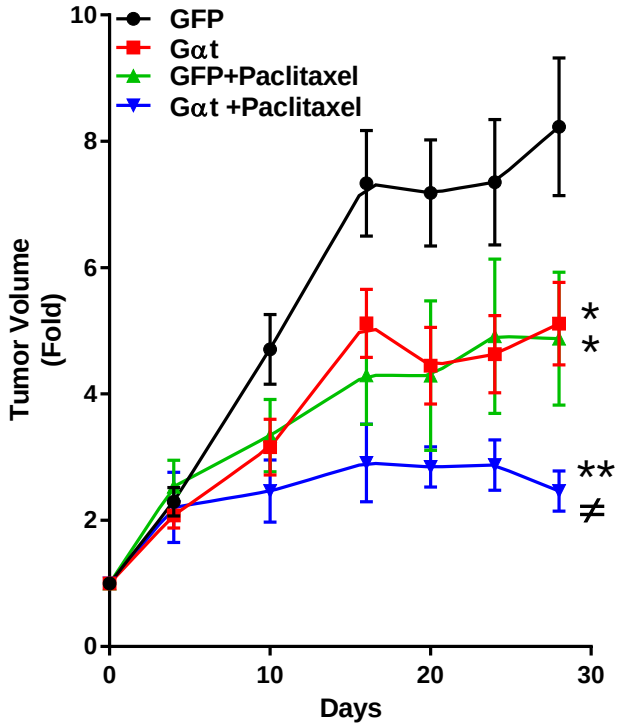

Fig 7. Blocking Gβγ signaling via induced Gαt expression increases the therapeutic efficacy of paclitaxel in PC3 xenografts (N=9-10). Mice were subcutaneously implanted with PC3 cells expressing inducible GFP or Gαt. Tumor growth was measured by caliper. When the tumors reached the size of ~300 mm^3, mice were fed with doxycycline-containing diets and treated with vehicle or paclitaxel (10mg/kg, i.p., twice per week). *, ** p<0.05 and 0.01 vs GFP; ≠ p<0.05 vs Gαt or GFP+paclitaxel.

Milestone-3: Data compilation and preparation of scientific presentation and manuscript for publication.
Accomplishments: we are submitting a manuscript summarizing the results of this study to *Oncotarget.*

4. Key research accomplishments:

- Establish the role of Gβγ signaling in promoting prostate cancer cell growth and metastasis in vitro and in vivo
- Demonstrate for the first time that blocking Gβγ signaling may be an efficacious approach for blocking tumor metastases in multiple tissues, in particular, bone.
- Elucidate the potential mechanisms by which Gβγ signaling mediates prostate cancer progression

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5. Conclusion:

In conclusion, our results provide compelling evidence for the critical role of Gβγ signaling in promoting prostate cancer progression, in particular, bone metastasis, and the efficacy of targeting Gβγ signaling to block cancer progression. These findings thus provide important support for the development of targeting Gβγ signaling as a novel therapeutic approach for prostate cancer.

6. Publications, Abstracts, and Presentations

Publications: a manuscript is in preparation for submission to *Oncotarget*

Abstracts: No

Scientific presentations:

2013: Invited symposium speaker for the Symposium "New roles for signaling by G protein βγ subunits" at the EB/ASPET meeting

2013: Department of Cell Biology and Physiology, Washington University School of Medicine, St Louis, MO. "Regulation of Gβγ signaling by WD40 proteins during directional cell migration"

2014: Department of Pharmacology, Georgia Regents University, GA. "GPCR and G protein signaling in cancer progression".

2015: Department of Physiology, Ohio State University, OH. "Function and regulation of GPCR and G protein signaling in cancer progression".

2015: Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University. "Function and regulation of GPCR and G protein signaling in cancer progression".

7. Inventions, Patents and Licenses: nothing to report

8. Reportable Outcomes: nothing to report

9. Other Achievements: No

10. References: No

Appendices: No