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Molecular Imaging of GSK3 β and CK1 α kinases

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Abstract

Glycogen synthase kinase-3 (GSK3 β) and casein kinase-1 alpha (CK-1 α) are multifunctional kinases that play critical role in the regulation of a number of cellular processes. In spite of their importance, molecular imaging tools for non-invasive and real-time monitoring of their kinase activities have not been devised. Here, we report development of bioluminescent GSK3 β and CK-1 α reporter (BGCR) based on firefly luciferase complementation. Treatment of SW620 cells stably expressing the reporter with inhibitors of GSK3 β (SB415286 and LiCl) or CK1 α (CKI-7) resulted in dose and time dependent increase in BGCR activity which were validated using western blotting. No increase in bioluminescence was observed in case of S37A mutant (GSK3 β inhibitors) or with S45A mutant (CKI-7) demonstrating the specificity of the reporter. Imaging of mice tumor xenograft generated with BGCR expressing SW620 cells following treatment with LiCl showed unique oscillations in GSK3 β activity which were corroborated by phospho-GSK3 β immunoblotting. Taken together, BGCR is novel molecular imaging tool that reveals unique insight into GSK3 β and CK1 α kinase activities and may provide powerful tool in experimental therapeutics for rapid optimization of dose and schedule of targeted therapies and for monitoring therapeutic response.

Keywords

Molecular imaging; GSK3 β ; CK1 α ; split luciferase; reporter

Introduction

GSK3 is a ubiquitously expressing multifunctional serine/threonine kinase which was originally identified in mammals as a critical mediator in glycogen metabolism and insulin

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signaling. It is now known that GSK3 is a key regulator of diverse signaling pathways involved in the regulation of glycogen metabolism, protein synthesis, cell mobility, cell fate, proliferation and survival [1;2]. Two GSK3 isoforms (GSK3 α and GSK3 β) are present in mammals which are encoded by two distinct genes located on different chromosomes. GSK3 α and GSK3 β are not functionally identical and redundant though they show a high degree of similarity (85%) [3]. The signaling pathway and protein function of GSK3 β are much better investigated. GSK3 β is active in resting cells and is inactivated during cellular responses. Due to its diverse cellular functions, the pathways in which GSK3 β acts as a key regulator, when dysregulated, have been implicated in the development of a number of human diseases such as diabetes, some neurodegenerative diseases, bipolar disorder, cardiovascular disease, tumorigenesis and cancer progression [1;2;3;4;5]. GSK3 β has preference for 'primed' substrates [6] and more than 40 proteins have been described as substrates of GSK3 β , a number of them have been implicated in tumorigenesis like β -catenin, APC, AXIN, and p53.

Casein kinase-1 group (CK1) enzymes have been implicated in a variety of biological functions, including Wnt pathway regulation [7;8], circadian rhythm [9], chromosome segregation [10;11], spindle formation [12;13;14], nuclear import [15], cell cycle regulation [16] and apoptosis [17]. Deregulation of CK1 isoforms has been described in neurodegenerative diseases [18], Alzheimer [19], sleeping disorders [20] and in cancer [21]. GSK3 β and CK1 α have some common target protein like β -catenin and p53 [22] but do not share target sites.

β -catenin is phosphorylated at the N-terminus by CK1 α at S45, and T41, S37 and S33 by GSK3 β [23;24] and marks it for ubiquitin-mediated degradation. Presence of ligand inactivates GSK3 β and prevents it from phosphorylating β -catenin, thus stabilizing β -catenin in the cytoplasm. As β -catenin accumulates, it translocates into the nucleus where it binds to TCF/LEF family of transcription factors and turn on a number of target genes [25;26]. In human malignancies such as cancers of colon, liver and skin, inappropriate activation of the Wnt/ β -catenin signaling pathway has been shown to occur through gain-of-function mutations resulting in the loss of GSK3 β and CK1 α phosphorylation site in β -catenin.

Most of these studies utilize conventional modalities, such as western blotting, immunoprecipitation and other modalities which provide an excellent molecular snapshot image but fail to capture the dynamic variance in the signaling cascade. Here we report development of a genetically engineered chimeric reporter which monitors GSK3 β and CK1 α activity in real time non-invasively.

The bioluminescent GSK3 β and CK-1 α reporter (BGCR) is generated after a luciferase complementation based reporter [27]. BGCR is a sensitive, dynamic and quantitative sensor of both GSK3 β and CK1 α which responds in a dose and time dependent manner to the inhibition of these kinases. In a tumor xenograft mice model, BGCR reveals oscillatory changes in activity of GSK3 β in response to LiCl. Such real time understanding of the kinase activity was not possible without the molecular imaging sensors for kinases. In summary this reporter represent a unique tool for sensitive real-time imaging of GSK3 β and CK1 α kinase activities and has the potential for use in enhancing our understanding of the biology of these key kinase regulators in living subjects and cells as well as for screening, characterization and pharmacodynamic analysis of potential regulators and drugs that target the GSK3 β and CK1 α .

Results

Construction of BGCR

To image GSK3 β kinase activity, we constructed a recombinant chimeric reporter (Figure 1A) consisting of a 20 amino acid target peptide sequence (derived from β -catenin (aa 29-48, [26]) which is a substrate for GSK3 β and CK1 α [28]. The BGCR reporter also consisted of the FHA2 phospho-serine/threonine binding domain of Rad53 [29], flanked by N-Luc (residues 1 to 416 of firefly luciferase) and C-Luc (residues 398 to 550 of firefly luciferase)[30]. The functional basis (Figure 1B) of the reporter was that when kinase was active, phosphorylation of the substrate peptide would result in the binding of the phospho-serine/threonine residue to the FHA2 domain, thus preventing reconstitution of N-Luc with C-Luc due to steric hindrance. Inhibition of kinase activity would result in loss of phosphorylation of the substrate peptide, thus releasing it from interaction with the FHA2 phospho-peptide binding domain. In this situation, N-Luc and C-Luc can associate and reconstitute luciferase activity, which can be detected by bioluminescence imaging.

Treatment with GSK3 β inhibitors indicate high sensitivity of BGCR reporter

To investigate if BGCR senses inhibition of GSK3 β and CK1 α , stable cell lines expressing the reporter were constructed. SW620 colon carcinoma cells expressing the reporter were treated with the small molecular inhibitor SB415286 and LiCl, agents that have been shown to inhibit GSK3 β [31;32;33;34], as well as CKI-7, a CK1 α inhibitor.

When SW620 cells stably expressing the WT reporter was treated with SB415286, a GSK3 inhibitor, a dose dependent increase in bioluminescence was observed. Quantitation of bioluminescence after treatment with 1-50 μ M of SB415286 revealed a dose and time dependent increase in BGCR-WT activity (Figure 1C). The increase in bioluminescence was rapid which peaked between 30 min to 1 h (Figure 1C'). Influence of the SB415286 on phosphorylation of the substrate protein was confirmed by antibodies to phosphorylated S33-S37-T41 β -catenin. A dose dependent decrease in phospho- β -catenin and concomitant increase in total β -catenin was observed (Figure 1D). No significant change in levels of phosphorylated S45 β -catenin, total GSK3 β and GAPDH was observed during the treatment. Additionally, expression of the BGCR-WT reporter, as detected by anti-luciferase antibody, did not change for the doses and time points tested. Therefore, the increase in BGCR-WT bioluminescence is based on changes in reporter conformation.

In response to increasing doses of LiCl (0-60 mM) BGCR-WT expressing SW620 cells had a 1.5-7.5 fold increase in bioluminescence activity over vehicle treated cells in a dose dependent manner (Figure 1E) over an eight hour period. To confirm if the observed changes in BGCR-WT activity correlated with changes in GSK3 β kinase activity, representative samples were analyzed by western blot analysis. As shown in figure 1F, levels of phospho S33-S37-T41 β -catenin were found to decrease over time in response to 50 mM LiCl, without a significant change in phosphorylated S45 β -catenin and total β -catenin levels. To measure GSK3 β activity we utilized an antibody that is specific for the inhibitory Ser-9 phosphorylation within GSK3 β . As shown in figure 1c, a time dependent increase in signal was observed without a significant change in levels of total GSK3 β or CK1 α . In addition, expression levels of the reporter also remained unchanged indicating that changes observed in bioluminescence activity were due to changes in activity and not due to changes in expression levels.

Validation of BGCR reporter using CK1 α inhibitor

We designed the reporter such that the target peptide sequence present within the BGCR contains phosphorylation sites for GSK3 β as well as CK1 α . Therefore, the BGCR-WT

reporter was also validated by treating with CKI-7, a small molecular weight inhibitor specific for CK1 α . In response to treatment with CKI-7 a dose and time dependent increase in SW620 BGCR-WT bioluminescence activity was observed (Figure 2A). Western blot analysis of representative samples revealed a time dependent decrease in phosphorylation of β -catenin at S45 a known target of CK1 α (Figure 2B). A decrease in phosphorylated S33-S37-S41 β -catenin was also observed after 2 h. No significant change in levels of total β -catenin, CK1 α and GSK3 β as well as the BGCR-WT reporter was observed. A similar decrease in phosphorylation of β -catenin at S45 was observed with increasing doses of CKI-7 without a significant change in BGCR, CK1 α and GSK3 β (Figure 2B). To determine whether the inhibitor mediated increase in BGCR-WT bioluminescence activity was due to decrease in phosphorylation of target peptide within the reporter, we immunoprecipitated the BGCR-WT with anti-luciferase antibody. Western blotting of these samples with a phospho(S33-S37-T41)- β -catenin antibody suggested a decrease in phosphorylation of target peptide within the reporter (Figure 2C).

Validation of specificity of the BGCR reporter using mutant reporters

We constructed a series of mutant reporters (Figure 1A) to dissect the role of GSK3 β and CK1 α target sites in β -catenin derived peptide in BGCR. Based on reports that a single mutation was sufficient to abolish β -catenin phosphorylation by GSK3 β [35], the residue corresponding to S37 of β -catenin was mutated to the neutral amino acid alanine within the reporter (BGCR S37A). Additionally, the CK1 α target site corresponding to S45 within β -catenin was also independently mutated to Ala (BGCR S45A). Lastly, a control reporter was constructed wherein each of the relevant target phosphorylation sites (S33, S37, T41 and S45) were mutated to Ala (BGCR QUAD). Stable cell lines expressing each of these three reporters were generated in SW620 colon carcinoma cells. Treatment of wild-type BGCR and BGCR S45A expressing cells with LiCl (50 mM) resulted in a time dependent increase in bioluminescence activity (Figure 3A). In contrast, the BGCR S37A and the BGCR QUAD expressing cells did not exhibit a significant change in reporter activity (Figure 3A). Western blot analysis of the BGCR S45A cells demonstrated a time dependent increase in phospho(S9)-GSK3 β levels (Figure 3B) and a corresponding decrease in levels of phospho(S33-S37-T41)- β -catenin but not in phospho(S45)- β -catenin or in CK1 α .

To investigate the specificity of the reporter for CK1 α , the above four reporter expressing cells were treated with CKI-7 (250 μ M). Bioluminescence activity measurements revealed a time dependent increase in BGCR wild-type and BGCR S37A expressing cells, while the BGCR S45A and the BGCR QUAD cells did not have a time dependent change in bioluminescence activity (Figure 3C). Western blot analysis of the CKI-7 treated cells revealed a time dependent decrease in phosphorylation of S45 β -catenin (Figure 3D), while no significant change in levels of total β -catenin, GSK3 β or CK1 α was observed. Similar level of reporter expression in stable cell lines was confirmed by western blotting (Figure 3E). Measurement of basal bioluminescence exhibited between 2-3 folds increase in S37A, S45A and QUAD mutant reporters over WT which was an expected result as replacement of target Ser/Thr with Ala in mutant reporters would mimic inhibition of phosphorylation (Figure 3E).

To determine whether the increase in BGCR S45A bioluminescence activity was due to decrease in phosphorylation of target peptide within the reporter, we immunoprecipitated the BGCR S45A with anti-luciferase antibody. Western blotting of these samples with a phospho(S33-S37-T41)- β -catenin antibody suggested a decrease in phosphorylation of target peptide within the reporter (Figure 3F).

Measurement of GSK3 β kinase activity in response to Wnt3a

To monitor BGCR activity with a biological transducer of Wnt signaling, we treated SW620 BGCR WT cells with Wnt condition media obtained from Wnt3a secreting mouse fibroblast cell line (L-wnt3a cells; [36]). No Change in reporter activity was observed in SW620 BGCR. This corroborates existing literature that mutations of APC and/or Axin cause constitutive activation of Wnt-signaling and leads to desensitization of treatment with Wnt [37;38]. Therefore, we created stable lines in HEK293 cells and characterized BGCR response to Wnt.

Upon treatment with Wnt3a conditioned media, more than a 2 fold induction in BGCR bioluminescence activity was observed with BGCR WT reporter cell line which remained active for 3 h (Figure 4A). HEK293 cells stably expressing S37A mutant reporter did not exhibit any induction of BGCR activity (Figure 4A). Cells treated similarly and harvested for western blotting indicated a decrease in phospho(S33-S37-T41)- β -catenin levels and increase in total β -catenin levels (Figure 4B). Further, an increase in inhibitory GSK3 β phosphorylation at S9 was detected. Levels of total GSK3 β and CK1 α were not found to change with the treatments. Anti-luciferase blot confirms that a change in bioluminescence activity was due to conformational change and not due to change in reporter expression (Figure 4B).

Molecular imaging of GSK3 β activity reveals oscillatory modulations *in vivo*

Having established the specificity and sensitivity of BGCR reporter in-vitro, we next investigated if BGCR activity can be used as surrogate marker for GSK3 β and CK1 α activities in-vivo. Tumor xenograft of SW620 BGCR WT cells were generated in athymic nude mice and once the tumor had grown to 40-60 mm³, animals were subjected to bioluminescence imaging to record the basal bioluminescence. Three hours later, animals were injected i.p. with a single dose of 400mg/kg body weight with LiCl. This dose was in accordance with a previous report to inhibit GSK3 β activity [39]. Bioluminescence imaging of the animals was performed at 1 h following treatment and continued for 34 h with 3 h interval between imaging. Representative images at pretreatment and post treatment with either LiCl or vehicle control (saline) at 1 h, 4 h, 7 h and 13 h are shown in Fig. 5.

Quantization of the bioluminescence averaged over 5 animals (10 tumors) revealed an increase in bioluminescence after 1 h (Figures 5A, B) which peaked at 7 h (greater than 4 folds) (Figure 5B). A second peak was then observed at 25 h (greater than 3 folds). Mice injected with saline (10 tumors) did not show a noticeable increase in bioluminescence during the 34 h period. These data indicate that GSK3 β levels oscillate in vivo xenograft models. Since this reporter senses inhibition of GSK3 β and phosphorylation at S9 has been reported in literature as an inhibitory post translational modification, we next evaluated correlation between phosphorylation at S9 in GSK3 β and bioluminescence data. Towards this, tumors from SW620 BGCR WT xenografts bearing animals were harvested at the time points similar to bioluminescence imaging and subjected to western blotting using phospho(S9)-GSK3 β antibody (Figure 5C). When the phospho(S9)-GSK3 β reactivity was quantitated, a correlation between the levels of S9 phosphorylated GSK3 β in tumor lysates and bioluminescence of BGCR activity was observed (Figure 5D). We attempted to measure CK1 α activity in our tumor xenograft model but due to a lack of in-vivo compatible CK1 α inhibitor, these experiments were not possible.

Discussion

Conventional biochemical assays for evaluation of signaling events only provide a “snapshot” view of the complex and dynamic activity of the pathway under investigation. It

is increasingly becoming clear that signaling pathways are modulated at multiple levels, including positive and negative feedback loops. To obtain a true measure of the activity of a given signaling pathway, assays that take into consideration the temporal dynamics of the pathway as well as the impact of multiple regulatory molecules on the ultimate consequence of the signaling activity are required. Important role played by GSK3 β and CK1 α in multiple pathways including signal dependent phospho-regulation of β -catenin is one such example. The ability to non-invasively and in real-time evaluate the activity of these two key kinases would significantly enhance our understanding of their biology. To this end, we here describe the construction of four reporter molecules which can be used to evaluate the activation or inhibition of GSK3 β and CK1 α either independently or in combination.

The WT reporter contained the N-terminal phosphorylation domain of β -catenin that is responsive to phosphorylation by CK1 α and GSK3 β . The ability of the WT reporter to respond to treatment by SB415286, LiCl and CKI-7 in a time and dose dependent manner validated the specificity of the reporter for both kinases. In contrast the S37A mutant reporter wherein the key GSK3 target site was mutated to alanine only responded to CKI-7 but not to LiCl further demonstrating its specificity for sensing CK1 α activity. Similarly, the S45A mutant wherein the CK1 α target site was mutated to alanine failed to respond to CKI-7 but was responsive to LiCl, a modulator of GSK3 β . In addition to having the expected specificity, these reporters demonstrated that bioluminescence activity could be used as a surrogate for each of the regulatory kinases in a dose and time dependent manner as demonstrated by the high degree of correlation observed between changes in photon counts and with changes in levels of target phospho-proteins.

Utilizing an elegant genetic approach and a plethora of distinct phospho- β -catenin antibodies, Liu et al., reported that for phosphorylation of β -catenin by GSK3 at the S33, S37 and T41, the S45 first has to be phosphorylated by CK1 [28]. This concept of priming kinase activity was later supported by a number of reports [1;40;41;42;43;44;45]. However, Wang et al., suggested that the S45 primer model for GSK3 β is not universally applicable especially in human colon malignancies [46]. Interestingly, comparison of our data with BGCR activity and conventional western blotting using the phospho specific β -catenin antibodies suggest that loss of casein kinase phosphorylation site within the BGCR (S45A mutant) does impede its ability to sense inhibition by GSK3 β inhibitors (Figure 3A). These results suggest that GSK3 β mediated phosphorylation of β -catenin peptide in BGCR may not require priming by CK1 α . However, monitoring the levels of phosphorylated β -catenin by western blotting suggest that a decrease in levels of phosphorylated S33-S37-T41- β -catenin is always observed with CKI-7 treatment (Figure 2B and 3D). Although, such observation suggests requirement of priming kinase activity for GSK3 β , it does not rule out off target effects of CKI-7 and/or CKI-7 mediated changes in kinetics of phosphatases involved in dephosphorylation of β -catenin.

GSK3 β is a unique kinase involved in regulating a number of distinct signaling pathways involved in processes ranging from embryogenesis to cell death [1]. Time lapse monitoring of inhibition of GSK3 β activity following a single dose of LiCl showed an oscillatory modulation of kinase activity in tumor xenograft model (BGCR-WT). A peak in bioluminescence activity suggesting maximal inhibition was observed at 7 h after LiCl administration which correlated with an increase in levels of phospho(S9)-GSK3 β (inactive form of GSK3). This was an expected result based on existing literature on LiCl mediated inhibition of GSK3 β in experimental animals [47] and published plasma half life of LiCl in mice [48]. Continued imaging of these animals revealed the emergence of a second bioluminescence peak at approximately 25 h after the initial dose of LiCl which was consistent with an increase in the intra-tumoral levels of phospho(S9)-GSK3 β as measured by immunoblotting (Figures 5C, D). A corresponding change in reporter activity was not

observed in untreated animals harboring BGCR-WT expressing tumors, indicating that the second peak in bioluminescence activity was due to the initial LiCl treatment. The second peak in BGCR activity subsided at 31 h post-LiCl treatment, which was also confirmed by a corresponding decrease in intra-tumoral levels of phospho (S9)-GSK3 β (Figures 5C, D). The oncological significance of such modulations is not clear yet and would require further investigations. However, similar oscillatory changes in GSK3 β have been reported during circadian rhythms [49;50;51].

BGCR provides a unique tool for real-time monitoring of dynamic changes in kinase activities of GSK3 β and CK1 α noninvasively. A significant advantage of such assays is that the molecular events in question are interrogated in their appropriate cellular environment (unlike *in vitro* assays) and under physiological conditions. Additionally, the cellular assays described here have the potential to select against compounds that are non-specifically cytotoxic as the reporter is turned on when GSK3 β or CK1 α activity is inhibited (Figure 1A). This unique property of the reporter offers an opportunity for high throughput screening for novel small molecule inhibitors while reducing the number of non-specific hits. Further, these cell based assays also impart information on cell permeability, stability and solubility of the compound. In addition to its role in cancer, deregulated expression of GSK3 β kinase is seen in innumerable human diseases such as, diabetes, schizophrenia, ADHD and Alzheimer disease [52; 53]. Therefore, use of BGCR in appropriate animal model will not only significantly enhance our understanding of the biology of cancer (and other diseases) but also allow investigation into efficacious therapeutic interventional modalities.

Materials and Methods

Construction of the reporter and generation of reporter expressing cell lines

The β -catenin substrate sequence (residues 29-47) harboring S45, T41, S37, S33 sites, flanked by linker (GGSGG) at each side was cloned into a pEF vector comprising split firefly luciferase and Rad53p FHA2 domain as described earlier [27] (Figure 1A). The primer sequences were as followed: BGCR wt forward primer CTAGAGGCGGTGGATCTTACCTGGACTCTGGTATTCACCTCGGGTGCAACCACAA CGGCGCCATCTTTATCGGGAAAGGGCGGTGGAC and BGCR wt reverse primer CCGGGTCCACCGCCCTTTCCCGATAAAGATGGCGCCGTTGTGGTTGCACCCGAG TGAATACCAGAGTCCAGGTAAGATCCACCGCCT. For generation of mutant reporters single primer mutagenesis protocol was used [54]. Primer sequences were as followed: S45A mutant GCAACCACAACGGCGCCAGCTTTATCGGGAAAGGGC, S37A mutant CCTGGACTCTGGTATTCACGCCGGCGCAACCACAACGGCGCC, and QUAD mutant CGGTGGATCTTACCTGGACGCTGGTATTCACCGGGTGCAACC~~CG~~CAACGGCGCC AGCTTTATCGGGAAAGGGC. All the clones were sequence verified. Colon cancer cell line SW620 and human embryonic kidney cells (HEK293) were obtained from ATCC and maintained in RPMI 1620 (Gibco-Invitrogen, Grand Island, NY) or DMEM respectively with 10% FBS. To generate stable cell lines expressing WT and mutant bioluminescent reporters, cells were transfected and selected in media containing 500 μ g/ml G418 (Gibco-Invitrogen, Grand Island, NY).

Live cell imaging and western blotting

Reporter cell lines were plated in 12 well plates and were treated with various doses of GSK3 β inhibitors SB415286 (Tocris Biosciences, Ellisville, MO), LiCl (Sigma Aldrich, St. Louis, MO) and CK1 α inhibitor CKI-7 (Toronto Research Chemicals, North York, Ontario, Canada) for indicated period of time and bioluminescence was acquired on IVIS 200 imaging platform (Caliper Life Science, Hopkinton, MA) after adding 100 μ g/ml D-

Luciferin (Xenogen Corp, Alameda, CA). ROI values were calculated for each exposure and analyzed. All the BLI measurements were done in triplicates. Data were derived from a minimum of three independent experiments.

Western blotting was done using routine protocols. Protein lysate was made in RIPA buffer containing 50mM tris-HCL, 1% NP-40, 0.25% deoxycholate-sodium salt, 150 mM NaCl, 1 mM EDTA, 1X protease and phosphatase inhibitors (Roche, Indianapolis) and loaded on SDS-PAGE gels and probed against phospho-(S33-S37-T41)- β -catenin, phospho-(S45)- β -catenin, total β -catenin, phospho-(S9)-GSK3 β , total GSK3 β , GAPDH (Cell Signaling Technology, Beverly, MA), CKI α (Santa Cruz Biotechnology, MA) and luciferase (Abcam, Cambridge, MA). Immunoprecipitation (IP) was carried out with 400 μ g total protein using antibodies raised against luciferase following routine protocol. Western blot intensity was measured using ImageJ v1.45 [55].

Tumor xenograft and *in vivo* bioluminescence imaging

All animal procedures were approved by the University of Michigan Committee for use and care of animals. Four to six weeks old athymic CD-1 male mice were procured from Charles River Laboratories (Wilmington, MA) and acclimatized for 4-5 days before use. The mice were injected with 2.5×10^6 cells stably expressing BGCR-WT reporter suspended in 50 μ l serum free RPMI on each flank and let grow until palpable tumors formed. Mice were given i.p. injection of 400 μ g/100 μ l of D-luciferin (Xenogen Corp., Alameda, CA.). Animals were anesthetized with isofluran, and imaged 5 min after administration of D-luciferin on Xenogen IVIS Spectrum system (Caliper Life Science, Hopkinton, MA) for up to 30 minutes. Background photon flux was measured 4 h before drug administration. Mice were injected intraperitoneally with 400 mg/kg body weight of LiCl (50 μ l of 200 mg/ml stock) or PBS and bioluminescence acquired after 1 h and every 3 h afterwards until 34 h.

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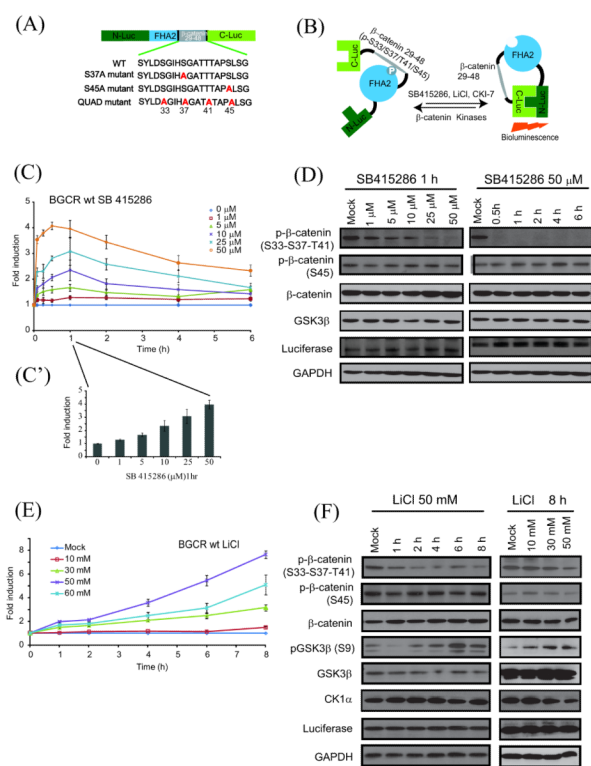
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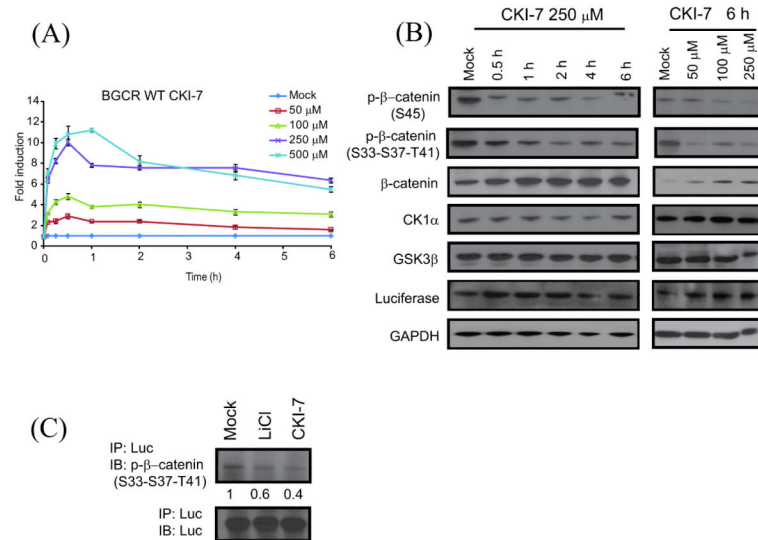
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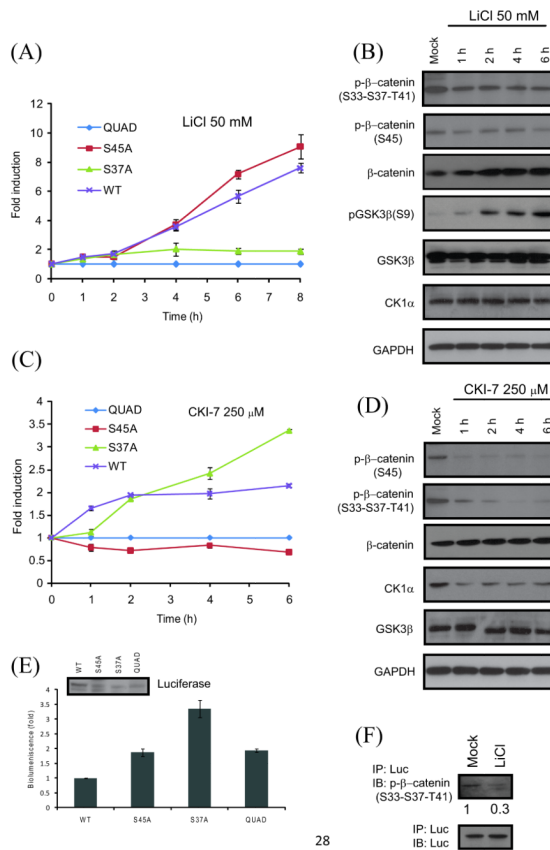
**Figure 1.**

Construction & validation of bioluminescent $GSK3\beta$ and $CK-1\alpha$ reporter (BGCR) and time and dose dependent imaging of $GSK3\beta$ activity. (A) The domain structure of the BGCR reporter. N-Luc and C-Luc are the amino- and carboxy-terminal domains of firefly luciferase that are fused to the appropriate ends of the reporter. The β -catenin peptide domain constitutes amino-terminal (aa 29-48) of $CK1\alpha$ and $GSK3\beta$ substrate sequence with a flexible GGSGG linker on either side. At the amino-terminal of the β -catenin peptide domain the yeast FHA2 phospho-ser/thr binding domain (residues 420-582) was appended. Three different mutant versions of the BGCR WT reporter were developed. The S37A mutant molecule contained Ser to Ala substitution at a $GSK3\beta$ phosphorylation site, S45A mutant where Ser to Ala substitution was done at the $CK1\alpha$ phosphorylation site and QUAD mutant where all four Ser/Thr within the substrate sequence were substituted to Ala. (B) The proposed mechanism of action for the BGCR reporter involves $CK1\alpha$ and $GSK3\beta$ dependent phosphorylation of β -catenin target peptide which results in its interaction with the FHA2 domain. In this form the reporter has minimal bioluminescence activity. In the absence of kinase activity, association of the N-Luc and C-Luc domains restores bioluminescence activity. (C) Cells stably expressing the BGCR-WT reporter were treated with various doses (1 μ M, 5 μ M, 10 μ M, 25 μ M, and 50 μ M) of SB415286 and BLI was measured serially from baseline to 6 h. The change in BLI activity over pretreatment (0 μ M) levels was determined and plotted as fold induction. BLI measurements were done in triplicates. Error bars denote SEM. (C') The bar diagram shows dose dependent changes in reporter activity at 1 h with SB415286. (D) Western blot analysis of samples from Fig 1C using anti-phospho-(S33-S37-T41), phospho-(S45)- β -catenin, total β -catenin, $GSK3\beta$, reporter (luciferase) and GAPDH antibodies. (E) SW620 cells stably expressing reporter were treated in triplicates with various doses of LiCl (10 mM, 30 mM, 50 mM and 60 mM) and BLI was measured serially until 8 h. The change in BLI activity over vehicle control treated (mock) levels for each time point was calculated and plotted as fold change. All the BLI measurements were done in triplicates. Error bars denote SEM. (F) Western blot analysis of the cells stably expressing

BGCR-WT reporter were treated with 50 mM LiCl for 1 h, 2 h, 4 h, 6 h and 8 h and various doses of LiCl (10 mM, 30 mM, and 50 mM) for 8 h using anti-phospho-(S33-S37-T41)- β -catenin, phospho-(S45)- β -catenin, total β -catenin, phospho-(S9)-GSK3 β , total GSK3 β , CK1 α , luciferase and GAPDH antibodies.

**Figure 2.**

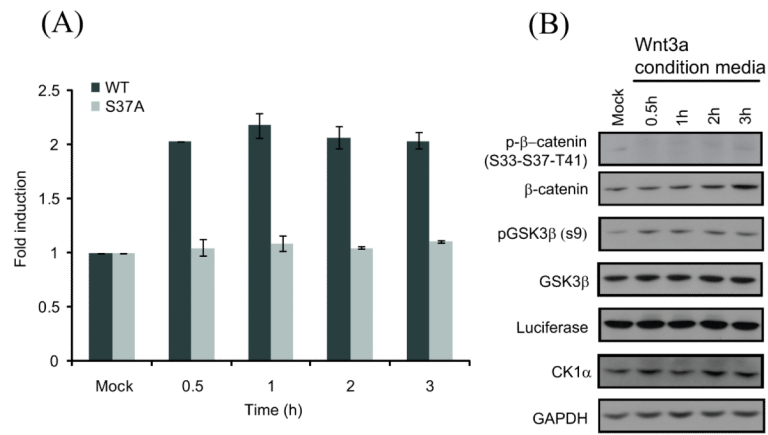
Measurement of CK1 α activity in a dose and time dependent manner. **(A)** SW620 cells stably expressing the BGCR-WT reporter were treated with various doses (50 μ M, 100 μ M, 250 μ M, and 500 μ M) of the CK1 α inhibitor CKI-7 and BLI was measured until 6 h. The change in BLI activity over DMSO control treated (mock) levels was determined and plotted as fold induction. BLI measurements were done in triplicates. Error bars denote SEM. **(B)** Western blot analysis of the cells stably expressing the BGCR-WT reporter were treated with 250 μ M CKI-7 for 0.5 h, 1 h, 2 h, 4 h, and 6 h and various doses of CKI-7 (50 μ M, 100 μ M, and 250 μ M) for 1 h using anti-phospho-(S33-S37-T41)- β -catenin, phospho-(S45)- β -catenin, total β -catenin, CK1 α , total GSK3 β , luciferase and GAPDH antibodies. **(C)** BGCR-WT reporter expressing cells were treated with 50 mM LiCl or 250 μ M CKI-7 for 6 h. Luciferase specific antibody was used to immunoprecipitate the BGCR reporter molecule and western blot analysis was performed using phospho-(S33-S37-T41)- β -catenin antibody, as well as luciferase specific antibody as control.



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Figure 3.

Confirmation of the BGCR reporter activity using mutant reporters. **(A)** SW620 cells stably expressing WT, S37A, S45A and QUAD mutant reporters were treated with 50mM LiCl and BLI was measured at 1h, 2h, 4h, 6h and 8h. The change in BLI activity over QUAD levels was determined and plotted as fold induction. BLI measurements were done in triplicates. Error bars denote SEM. **(B)** SW620 cells stably expressing S45A mutant were treated with 50 mM LiCl for 1 h, 2 h, 4 h and 6 h and western blot analysis was carried out using antibodies raised against phospho-(S33-S37-T41)-β-catenin, phospho-(S45)-β-catenin, total β-catenin, phospho-(S9)-GSK3β, total GSK3β, CK1α and GAPDH. **(C)** Cells stably expressing WT, S37A, S45A and QUAD mutant reporters were treated with the CK1α inhibitor CKI-7 (250 μM) and BLI was measured at 1 h, 2 h, 4 h, and 6 h. The change in BLI activity over QUAD levels was determined and plotted as fold induction. BLI measurements were done in triplicates. Error bars denote SEM. **(D)** SW620 cells stably expressing S45A mutant reporter were treated with 250 μM CKI-7 for 1 h, 2 h, 4 h and 6 h and western blot analysis was carried out using antibodies raised against phospho-(S33-S37-T41)-β-catenin, phospho-(S45)-β-catenin, total β-catenin, CK1α, total GSK3β, and GAPDH. **(E)** Basal bioluminescence was calculated for SW620 cells stably expressing WT, S37A, S45A and QUAD mutant reporters without any treatment. Expression of reporter in each stable cell line is shown with antibody against luciferase. **(F)** S45A mutant reporter expressing cells were treated with 50 mM LiCl for 6 h. Luciferase specific antibody was used to immunoprecipitate the BGCR reporter molecule and western blot analysis was performed using phospho-(S33-S37-T41)-β-catenin antibody, as well as luciferase specific antibody as control.

**Figure 4.**

Measurement of Wnt modulated change in GSK3 β activity. **(A)** HEK293 cells stably expressing the BGCR-WT and S37A mutant reporters were treated with the Wnt-conditioned media for 0.5 h, 1 h, 2 h, and 3 h and BLI activity was measured. The change in BLI activity over control levels was determined and plotted as fold induction. Error bars denote SEM. **(B)** HEK293 cells stably expressing the BGCR WT reporter were treated with the Wnt-conditioned media for 0.5 h, 1 h, 2 h, and 3 h and western blot analysis was carried out using antibodies to phospho-(S33-S37-T41)- β -catenin, total β -catenin, phospho-(S9)-GSK3 β , total GSK3 β , luciferase, CK1 α and GAPDH.

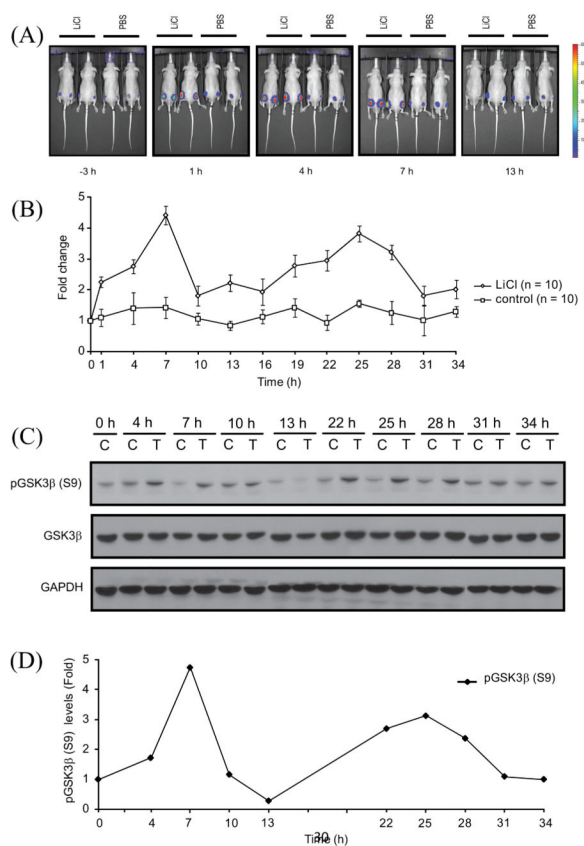


Figure 5. Imaging of GSK3 β activity *in vivo*. **(A)** CD-1 nude mice were subcutaneously implanted with SW620 cells stably expressing the BGCR-WT reporter. BLI activity was monitored in mice after 2 weeks when tumors reached about 40-60 mm³ size. BLI activity of pretreated and treated with vehicle control (PBS) and LiCl (400mg/kg) was monitored at 3 h interval till 34 h. Representative BLI images at -3 h, 1 h, 4 h, 7 h, and 13 h were shown. All window and level settings were consistent for all the acquisition. **(B)** ROI were drawn manually above the tumors and peak bioluminescence was measured until 34 h post treatment. BLI activity was expressed as fold induction as means \pm SEM for each of the groups, based on pretreatment level. **(C)** CD-1 nude mice harboring xenograft of BGCR-WT expressing cells were treated with vehicle control PBS (control, C) or treated with 400mg/kg LiCl (treated, T) and tumors were harvested at times points mentioned and western blot analysis was done with pGSK3 β (S9), total GSK3 β and GAPDH antibodies. **(D)** Densitometric analysis of pGSK3 β (S9) western blot for vehicle treated and LiCl treated tumor was done and shown as fold change from control levels at each time point.