Short Communication

Transcriptional Regulation of SULT1C2 by Vitamin D Receptor in LS180 Human Colorectal Adenocarcinoma Cells

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Running Title: Vitamin D regulation of SULT1C2

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Abbreviations: DR3, direct repeat of AGGTCA with 3 intervening nucleotides;

GW3965, 3-[3-[N-(2-chloro-3-trifluoromethylbenzyl)-(2,2-

diphenylethyl)amino]propyloxy]phenylacetic acid; GW4064, 3-(2,6-dichlorophenyl)-4-(3'-

carboxy-2-chlorostilben-4-yl)oxymethyl-5-isopropylisoxazole; HEK, human embryonic

kidney cells; Luc, firefly luciferase; PCR, polymerase chain reaction; PXR, pregnane X

receptor; PXRE, PXR response element; RXR, retinoid X receptor; SULT, cytosolic

sulfotransferase; VitD$_3$, 1α,25-dihydroxyvitamin D$_3$; VDR, vitamin D receptor; VDRE,

vitamin D response element; WT, wild-type.

Abstract

The factors that regulate expression of genes in the 1C family of human cytosolic sulfotransferases (SULT1C) are not well understood. In a recent study evaluating the effects of a panel of transcription factor activators on SULT1C family member expression in LS180 human colorectal adenocarcinoma cells, we found that SULT1C2 expression was significantly increased by 1α,25-dihydroxyvitamin D_3 (VitD$_3$) treatment. The objective of the current study was to identify the mechanism responsible for VitD$_3$-mediated activation of SULT1C2 transcription. VitD3 treatment of LS180 cells activated transcription of a transfected luciferase reporter plasmid that contained ~5Kb of the SULT1C2 gene, which included 402 nt of the non-coding exon 1, all of intron 1, and 21 nt of exon 2. Although computational analysis of the VitD$_3$-responsive region of the SULT1C2 gene identified a pregnane X receptor (PXR)-binding site within exon 1, the transfected 5Kb SULT1C2 reporter was not activated by treatment with rifampicin, a prototypical PXR agonist. However, deletion or mutation of the predicted PXR-binding site abolished VitD$_3$-mediated SULT1C2 transcriptional activation, identifying the site as a functional vitamin D response element (VDRE). We further demonstrated that vitamin D receptor (VDR) can interact directly with the SULT1C2 VDRE sequence using an ELISA-based transcription factor binding assay. In conclusion VitD$_3$-inducible SULT1C2 transcription is mediated through a VDRE in exon 1. These results suggest a role for SULT1C2 in VitD$_3$-regulated physiological processes in human intestine.

Introduction

The cytosolic sulfotransferases (SULTs) are a family of conjugating enzymes that catalyze the biotransformation of a wide variety of exogenous and endogenous substrates. The human SULT1C subfamily consists of three members, SULT1C2, SULT1C3, and SULT1C4. The characterization of SULT1C substrate specificity, expression, and regulation is incomplete, but available evidence suggests that these enzymes might play metabolic roles during development (Runge-Morris and Kocarek, 2013). Human SULT1C2 is expressed in multiple tissues including fetal liver, stomach, and the vitamin D target tissues thyroid, intestine and kidney (Runge-Morris and Kocarek, 2013). SULT1C2 does not metabolize prototypical SULT substrates but has been shown to sulfonate thyroid hormones and some phenols and to bioactivate the procarcinogen N-hydroxy-2-acetylaminofluorene (Sakakibara et al., 1998; Li et al., 2000; Allali-Hassani et al., 2007).

We recently reported that treatment of LS180 human colorectal adenocarcinoma cells with several nuclear receptor activators, including GW3965 (liver X receptor agonist), GW4064 (farnesoid X receptor agonist), rifampicin [pregnane X receptor (PXR) agonist], or 1α,25-dihydroxyvitamin D_3 (VitD$_3$) [vitamin D receptor (VDR) agonist], increased SULT1C2 expression (Rondini et al., 2014). The VitD$_3$-mediated induction was especially pronounced and occurred at the mRNA, protein, and promoter activation levels. It therefore seems likely that VitD$_3$-mediated regulation of SULT1C2 could contribute to VitD$_3$-regulated processes in intestine and possibly other VitD$_3$ target tissues, such as kidney.

VitD$_3$ regulates gene transcription by activating the ligand-dependent nuclear receptor, vitamin D receptor (VDR), which binds as a heterodimer with retinoid X receptor (RXR) to vitamin D response elements (VDREs) in target genes (Haussler et al., 1997). VDREs are classically direct repeats of AGGTCA separated by three nucleotides (i.e., DR3 motifs). The objective of this study was to identify the *cis*-acting element responsible for VitD$_3$-mediated regulation of SULT1C2 transcription.

Materials and Methods

Cell Culture. LS180 colorectal adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured as previously described (Rondini et al., 2014). HEK 293 cells (human embryonic kidney cells), used for protein expression, were obtained from Dr. Ye-Shih Ho (Wayne State University) and cultured in Dulbecco's Modified Eagle Medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (all purchased from Life Technologies, Grand Island, NY). Cells were maintained under a humidified atmosphere of 95% air, 5% CO$_2$ at 37°C.

Preparation of Firefly Luciferase (Luc) Reporter Plasmids. Preparation of the SULT1C2 (-4998:-1)-Luc reporter plasmid has been described previously where it was referred to as SULT1C2#2 (Rondini et al., 2014). The SULT1C2 (-4827:-1)-Luc deletion construct was prepared using SULT1C2 (-4998:-1)-Luc as template, HotStar HiFidelity Polymerase (Qiagen, Valencia, CA), and the primer pairs listed in Supplemental Table 1. The PCR fragment was digested with KpnI and NheI and ligated into the corresponding sites of the pGL4.24 [luc2P/minP] reporter plasmid (Promega, Madison,

WI). Site-directed mutagenesis of a computationally predicted (MatInspector;

Genomatix, Inc., Cincinnati, OH) (Quandt et al., 1995; Cartharius et al., 2005) PXR-

binding site was performed using the the QuikChange II Kit (Agilent Technologies,

Santa Clara, CA) according to manufacturer's instructions, with SULT1C2 (-4998:-1)-

Luc as template and the primers listed in Supplemental Table 1.

Transient Transfection Analysis. Approximately 200,000 LS180 cells were

seeded into the wells of 12-well plates. Seventy-two hours later, cultures were

transfected with 1.6 μg of a SULT1C2 Luc reporter plasmid and 1 ng of the *Renilla*

luciferase expression plasmid, pRL-CMV (Promega), as previously described (Rondini

et al., 2014). After 18h, transfection medium was replaced and cells were treated with

0.1% ethanol (Sigma-Aldrich, St. Louis, MO) or 0.1 μM VitD$_3$ (Sigma-Aldrich) for 48h.

Cells were harvested for measurement of firefly and *Renilla* luciferase activities using

the Dual Luciferase Assay (Promega) and a GloMax Luminometer (Promega).

VDR•VDRE *In Vitro* Binding Assay. A VDR expression plasmid was prepared

by amplifying the VDR coding sequence (RefSeq NM_000376.2), using LS180 cDNA as

template, Herculase II Fusion DNA Polymerase (Agilent Technologies), and the primers

listed in Supplemental Table 1. The amplified fragment was digested with HindIII and

XhoI and ligated into the pcDNA3.1 expression plasmid (Life Technologies).

Approximately 1,000,000 HEK 293 cells were seeded into 100 mm plates. Once 70-

80% confluent, the cells were transfected with 60 μl Lipofectamine 2000 (Life

Technologies), 1.5 μg of VDR-pcDNA3.1, and 1.5 μg RXRα-pSG5 (provided by Dr.

Steven Kliewer, University of Texas Southwestern, Dallas, TX). Forty-eight hours later,

nuclear proteins were extracted using the NucBuster Protein Extraction kit (Novagen,

EMD Millipore, Billerica, MA), and protein concentrations were quantified using the

bicinchoninic acid protein assay (Thermo Fisher Scientific, Rockford, IL).

The Universal EZ-TFA Chemiluminescent Transcription Factor Assay (EMD

Millipore) was performed according to the manufacturer's protocol. Briefly, 0.75 µg HEK

293 nuclear extract containing VDR and RXRα, 2 pmol biotinylated capture probe

containing the VDRE consensus sequence from the rat osteocalcin gene [also known

as bone gamma-carboxyglutamate, official symbol Bglap; Markose et al., 1990; Demay

et al., 1992], and transcription factor assay buffer were added to the wells of a

streptavidin-coated microplate. Competition for binding of VDR to the capture probe was

assessed by adding one of the following unlabeled double-stranded oligonucleotides to

the binding reaction: (1) consensus VDRE; (2) mutated consensus VDRE (Gutierrez et

al., 2004); (3) SULT1C2 VDRE; (4) mutated SULT1C2 VDRE. The competitors were

added at 1.5- to 50-fold molar excess of the biotinylated capture probe. Background

binding was determined by assessing VDR binding to a biotinylated mutated consensus

VDRE probe. Capture probe, competitor probe, and negative control probe sequences

are listed in Supplemental Table 1. Capture probe-bound VDR was detected using the

primary antibody, VDR (D-6) X (sc-13133X; Santa Cruz Biotechnology, CA), diluted

1:5000, followed by horseradish peroxidase-conjugated secondary antibody (1:500

dilution), and chemiluminescence was measured using kit reagents and a GloMax

Luminometer. Data are expressed relative to VDR•RXR binding to the consensus VDRE

capture probe in the absence of competitor.

Statistical Analysis. Transfection and transcription factor binding assay data were analyzed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls or Dunnett's multiple comparison test. $p < 0.05$ was considered statistically significant. Binding curves with 95% confidence intervals were generated using the sigmoidal dose-response algorithm of Prism 6 for Windows (GraphPad Software Inc., La Jolla, CA).

Results and Discussion

We previously reported that $VitD_3$ treatment of LS180 cells increased activity from a transfected reporter plasmid containing ~5 Kb of the SULT1C2 gene (-4998:-1 relative to the translation start site in exon 2, shown schematically in Fig. 1) (Rondini et al., 2014). Recently, in a genome-wide ChIP-seq analysis of $VitD_3$-treated LS180 cells, Meyer et al. (2012) detected a VDR•RXR binding peak at nt 108,288,453 to 108,289,105 of chromosome 2 [Gene Expression Omnibus GSE31939; RefSeq NC_000002.12], with the peak center located within the SULT1C2 non-coding exon 1. This information suggested that a VDRE site is located near the 5'-end of our SULT1C2 (-4998:-1) fragment. We therefore deleted 171 nt from the 5'-end of SULT1C2 (-4998:-1), creating the SULT1C2 (-4827:-1)-Luc reporter. Figure 2 shows that this deletion abolished $VitD_3$-mediated SULT1C2 activation, confirming the presence of a $VitD_3$-responsive site in this region.

MatInspector software was used to identify putative transcription factor-binding sites within the deleted 171 nt sequence. Instead of detecting a prototypical VDR•RXR DR3 motif in exon 1, a motif identified as a putative PXR•RXR binding site was detected

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at nt -4887 to -4863 (predicted PXRE, Figure 1). However, we previously reported that treatment of LS180 cells with the prototypical PXR agonist, rifampicin, did not increase expression from the SULT1C2 (-4998:-1)-Luc reporter (Rondini et al., 2014), suggesting that the computationally-predicted sequence is not a functional PXR response element (PXRE), but rather potentially a VDRE site. Mutation of the core sequence of the predicted PXR-binding site (from GGT to AAC) within the SULT1C2 (-4998:-1)-Luc plasmid caused a 94% reduction in VitD$_3$-mediated SULT1C2 reporter activation compared to the wild-type construct (Figure 3), further supporting the conclusion that this site is a functional VDRE.

An ELISA-based transcription factor-binding assay was used to determine whether VDR•RXR can bind directly to the VDRE site in exon 1 of the human SULT1C2 gene. The capture probe containing the VDRE consensus sequence from the rat osteocalcin gene promoter was incubated with unlabeled competitor probes added in 50-fold molar excess and nuclear protein extract from HEK 293 cells expressing VDR and RXRα. The SULT1C2 VDRE competitor significantly inhibited VDR·RXR binding to the capture probe by 74%, which was similar to the inhibition (79%) that was observed when consensus VDRE competitor was added (Figure 4). Mutated consensus and SULT1C2 VDRE competitors did not significantly inhibit binding to the capture probe (25% and 31% inhibition, respectively). Incubation with increasing amounts (1.5- to 50-fold molar excess) of SULT1C2 VDRE competitor inhibited VDR•RXR binding to the capture probe with an IC$_{50}$ that was comparable to that of the consensus VDRE competitor (Figure 4 Inset).

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VDR and PXR are both members of the NR1I nuclear receptor group, and therefore have high sequence similarity (Kliewer et al., 1998). Additionally, they have been shown to recognize similar ligands and regulate similar target genes. For example, VDR and PXR are both activated by the secondary bile acid lithocholic acid (Staudinger et al., 2001; Makishima et al., 2002) and both receptors regulate expression of CYP3A4 (Bertilsson et al., 1998; Schmiedlin-Ren et al., 2001; Thummel et al., 2001). In the case of CYP3A4, PXR and VDR activate transcription through the same *cis*-acting elements, a proximal everted repeat with six intervening nucleotides motif and a distal DR3 motif (Goodwin et al., 1999; Thummel et al., 2001; Thompson et al., 2002). By comparison, while both PXR and VDR activate SULT1C2 expression in LS180 cells (Rondini et al., 2014), in this case the two receptors do not appear to utilize the same response element. While VitD$_3$ treatment activates SULT1C2 transcription through the element at nt -4887 to -4863, rifampicin treatment did not increase transcription from the (-4998:-1) reporter construct containing that element (Rondini et al., 2014). Therefore, VDR/PXR regulation of CYP3A4 differs somewhat from SULT1C2 regulation by these two receptors. Presumably the restriction by the SULT1C2 motif to VDR is physiologically important for ensuring appropriate SULT1C2 expression in response to hormonal versus xenobiotic signals. Further research is needed to determine the role that this mode of regulation plays in intestinal physiology.

Authorship Contributions

Participated in research design: Barrett, Kocarek, and Runge-Morris

Conducted experiments: Barrett and Fang

Performed data analysis: Barrett and Kocarek

Wrote or contributed to the writing of the manuscript: Barrett, Kocarek, and Runge-

Morris

References

Allali-Hassani A, Pan PW, Dombrovski L, Najmanovich R, Tempel W, Dong A, Loppnau P, Martin F, Thornton J, Edwards AM, Bochkarev A, Plotnikov AN, Vedadi M and Arrowsmith CH. (2007) Structural and chemical profiling of the human cytosolic sulfotransferases. *PLoS.Biol.* 5(5), e97.

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P and Berkenstam A. (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc.Natl.Acad.Sci.U.S.A.* 95(21), 12208-12213.

Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, and Werner T (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics.* 21 (13):2933-2942.

Demay MB, Kiernan MS, DeLuca HF and Kronenberg, HM. (1992) Characterization of 1,25-dihydroxyvitamin D3 receptor interactions with target sequences in the rat osteocalcin gene. *Mol.Endocrinol.* 6(4), 557-562.

Goodwin B, Hodgson E and Liddle C. (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol.Pharmacol.* 56(6), 1329-1339.

Gutierrez S, Liu J, Javed A, Montecino M, Stein GS, Lian JB and Stein JL. (2004) The vitamin D response element in the distal osteocalcin promoter contributes to chromatin organization of the proximal regulatory domain. *J.Biol.Chem.* 279(42), 43581-43588.

Haussler MR, Haussler CA, Jurutka PW, Thompson PD, Hsieh JC, Remus LS, Selznick SH and Whitfield GK. (1997) The vitamin D hormone and its nuclear receptor: molecular actions and disease states. *J Endocrinol.* 154:S57-S73.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T and Lehmann JM. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell.* 92(1), 73-82.

Li X, Clemens DL and Anderson RJ. (2000) Sulfation of iodothyronines by human sulfotransferase 1C1 (SULT1C1)*. *Biochem.Pharmacol.* 60(11), 1713-1716.

Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR and Mangelsdorf DJ. (2002) Vitamin D receptor as an intestinal bile acid sensor. *Science.* 296(5571), 1313-1316.

Markose ER, Stein JL, Stein GS and Lian JB. (1990) Vitamin D-mediated modifications in protein-DNA interactions at two promoter elements of the osteocalcin gene. *Proc.Natl.Acad.Sci.USA.* 87(5), 1701-1705.

Meyer MB, Goetsch PD, and Pike JW. (2012) VDR/RXR and TCF4/β-Catenin Cistromes in Colonic Cells of Colorectal Tumor Origin: Impact on c-FOS and c-MYC Gene Expression. *Mol Endocrinol.* 26(1):37-51.

Quandt K, Frech K, Karas H, Wingender E, and Werner T (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* 23 (23):4878-4884.

Rondini EA, Fang H, Runge-Morris M, and Kocarek TA. (2014) Regulation of cytosolic sulfotransferases 1C2 and 1C3 by nuclear signaling pathways in LS180 colorectal adenocarcinoma cells. *Drug Metab Dispos.* 42:361-368.

Runge-Morris M and Kocarek TA. (2013) Expression of the sulfotransferase 1C family: Implications for xenobiotic toxicity. *Drug Metab Rev.* 45(4):450-459.

Sakakibara Y, Yanagisawa K, Katafuchi J, Ringer DP, Takami Y, Nakayama T, Suiko M and Liu MC. (1998) Molecular cloning, expression, and characterization of novel human SULT1C sulfotransferases that catalyze the sulfonation of N-hydroxy-2-acetylaminofluorene. *J.Biol.Chem.* 273(51), 33929-33935.

Schmiedlin-Ren P, Thummel KE, Fisher JM, Paine MF and Watkins PB. (2001) Induction of CYP3A4 by 1 alpha,25-dihydroxyvitamin D3 is human cell line-specific and is unlikely to involve pregnane X receptor. *Drug Metab Dispos.* 29(11), 1446-1453.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH and Kliewer SA. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc.Natl.Acad.Sci.U.S.A.* 98(6), 3369-3374.

Thompson, P. D., Jurutka, P. W., Whitfield, G. K., Myskowski, S. M., Eichhorst, K. R., Dominguez, C. E., Haussler, C. A., and Haussler, M. R. (2002) Liganded VDR induces CYP3A4 in small intestinal and colon cancer cells via DR3 and ER6 vitamin D responsive elements. *Biochem.Biophys.Res.Commun.* 20;299(5), 730-738.

Thummel KE, Brimer C, Yasuda K, Thottassery J, Senn T, Lin Y, Ishizuka H, Kharasch E, Schuetz J and Schuetz E. (2001) Transcriptional control of intestinal cytochrome P-4503A by 1alpha, 25-dihydroxy vitamin D3. *Mol.Pharmacol.* 60(6), 1399-1406.

Footnotes

These results were presented in preliminary form at the Society of Toxicology 2015

Annual Meeting.

Figure Legends

Fig. 1. Schematic representation of the SULT1C2 (-4998:-1) fragment. A ~5Kb

fragment of the SULT1C2 gene containing nt -4998 to -1 relative to the translation start

site was amplified and ligated into a luciferase reporter plasmid. This fragment includes

402 nt of the non-coding exon 1, intron 1, and 21 nt of exon 2. Computational analysis

identified a putative PXR-binding site (predicted PXRE, core sequence underlined) at nt

-4887 to -4863. SULT1C2 (-4827:-1) shows the PXR-binding site deletion fragment.

**Fig. 2. VitD$_3$ treatment activates reporter expression from SULT1C2 construct (-
4998:-1) but not from deletion construct (-4828:-1) in LS180 cells.** LS180 cells were

transiently transfected with SULT1C2 (-4998:-1)-Luc, SULT1C2 (-4827:-1)-Luc, or

empty reporter plasmid (pGL4.24) and treated with 0.1% ethanol or 0.1μM VitD$_3$ for 48h.

Cells were harvested and luciferase activities were measured. Each bar represents the

mean ± S.D. of normalized (firefly/Renilla) luciferase activity relative to the activity

measured in ethanol-treated, SULT1C2 (-4998:-1)-Luc transfected cells (n=9 wells per

group, derived from combining data from three independent experiments with triplicate

transfection). ***Significantly different from ethanol-treated cells transfected with the

same reporter plasmid, *P < 0.001*.

**Fig. 3. Mutation of the predicted PXR-binding site in exon 1 attenuates VitD$_3$-
mediated SULT1C2 (-4998:-1)-Luc reporter activation.** LS180 cells were transiently

transfected with SULT1C2 (-4998:-1)-Luc containing either wild-type (WT) or mutated

(Mut) predicted PXR-binding site and treated with 0.1% ethanol or 0.1μM VitD$_3$. Forty-

eight hours later, the cells were harvested and luciferase activities measured. Each bar represents the mean ± S.D. normalized (firefly/*Renilla*) luciferase activity relative to the activity measured in ethanol-treated WT SULT1C2 (-4998:-1)-Luc transfected cells (n=6 wells per group, derived from combining data from two independent experiments with triplicate transfection). ***Significantly different from ethanol-treated control, *P < 0.001*.

Fig. 4. VDR•RXR binds to the predicted PXR-binding site (VDRE) in exon 1 of SULT1C2. An ELISA-based *In vitro* transcription factor-binding assay was performed as described in Materials and Methods. Binding reactions contained nuclear protein extract and a biotinylated consensus or mutated VDRE capture probe, alone or in the presence of a 50-fold molar excess of either wild-type (WT) or mutated (Mut) consensus VDRE or WT or Mut SULT1C2 VDRE competitor probe. Each bar represents the mean ± S.E.M. binding of VDR•RXR to a VDRE capture probe relative to the binding to the consensus VDRE capture probe that was detected in the absence of competitor (n=4, derived from means of four independent binding experiments, each performed with duplicate wells). ***Significantly different from consensus VDRE capture probe in absence of competitor, *P < 0.001*. Inset: Binding affinity was assessed by adding different amounts (1.5- to 50-fold molar excess) of competitor probes to the consensus VDRE capture probe and nuclear protein extract. IC_{50} values with 95% confidence intervals (CI) are shown. Each data point is the mean from two independent experiments, each performed in duplicate wells.
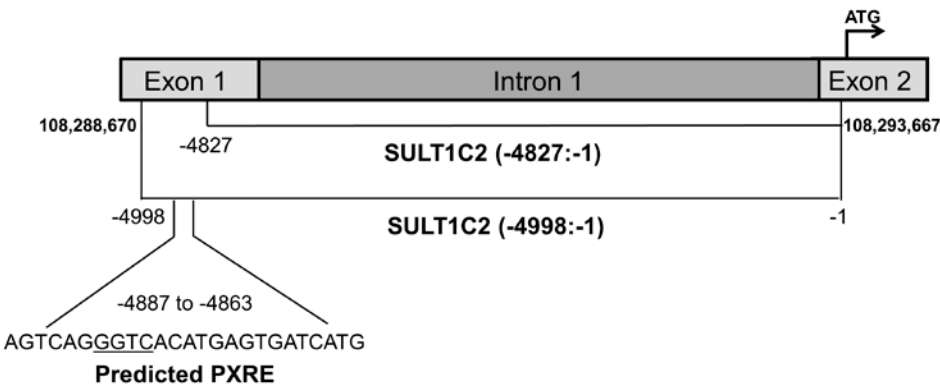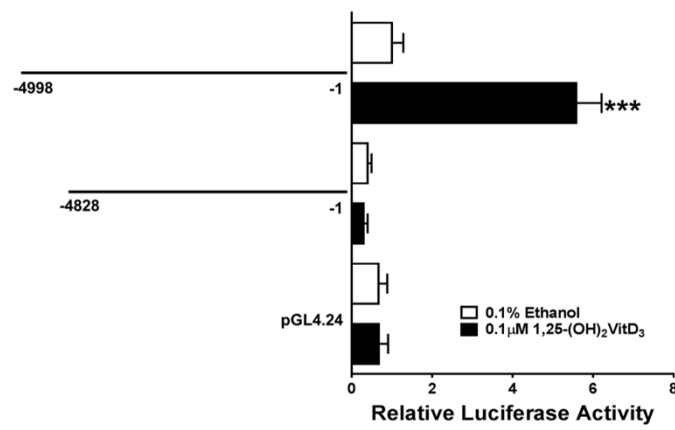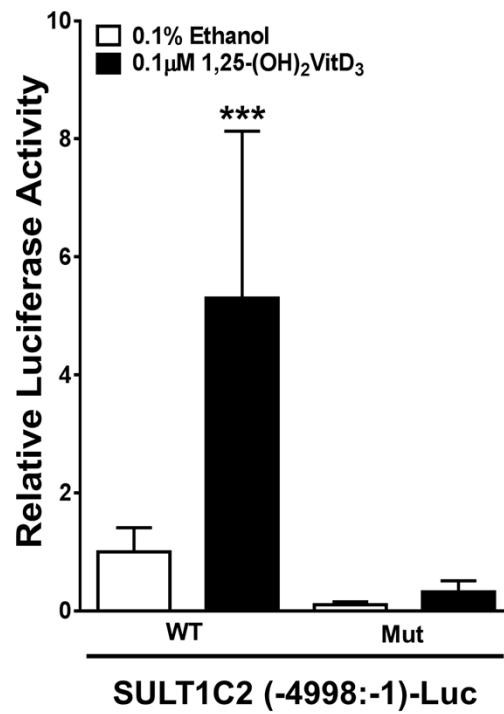
Fig. 1

Fig. 2

Fig. 3

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