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Title Page

Complete title: Induction of multidrug resistance transporter ABCG2 by prolactin in human breast cancer cells

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Running title page

Running title: Prolactin induces multidrug resistance transporter ABCG2

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Abbreviations

ABCG2, ATP-binding cassette, sub-family G, member 2; PRL, prolactin; PRLR, prolactin receptor, JAK2, Janus Kinase 2; STAT5, signal transducer and activator of transcription-5; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3-kinase; CISH, cytokine-inducible SH2-containing protein; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; FBS, fetal bovine serum; siRNA, short interfering RNA; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene; PD98059, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; LY294002, 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one; ERK1/2, extracellular signal-regulated kinases 1 and 2; AKT, protein kinase B; GH, growth hormone; ChIP, chromatin immunoprecipitation

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Abstract

The multidrug transporter, breast cancer resistance protein (BCRP, ABCG2), is upregulated in certain chemoresistant cancer cells and in the mammary gland during lactation. We investigated the role of the lactogenic hormone prolactin in the regulation of ABCG2. Prolactin dose-dependently induced ABCG2 in T-47D human breast cancer cells. This induction was significantly reduced by short interfering RNA (siRNA)-mediated knockdown of Janus Kinase 2 (JAK2). Knockdown or pharmacological inhibition of the down-stream signal transducer and activator of transcription-5 (STAT5) also blunted the induction of ABCG2 by prolactin, suggesting a role for the JAK2/STAT5 pathway in prolactin-induced ABCG2 expression. Corroborating these findings, we observed prolactin-stimulated STAT5 recruitment to a region containing a putative GAS element at -434bp upstream of the ABCG2 transcription start site. Introduction of a single mutation to the -434 GAS element significantly attenuated prolactin-stimulated activity of a luciferase reporter driven by the ABCG2 gene promoter and 5'-flanking region containing the -434 GAS motif. In addition, this GAS element showed strong copy number dependency in its response to prolactin treatment. Interestingly, inhibitors against the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) signalling pathways significantly decreased the induction of ABCG2 by prolactin without altering STAT5 recruitment to the GAS element. We conclude that the JAK2/STAT5 pathway is required but not sufficient for the induction of ABCG2 by prolactin.

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Introduction

The breast cancer resistance protein (BCRP or *ABCG2*) is the second member of the ABCG subfamily within ATP-binding cassette (ABC)-transporter superfamily. Classically associated with multidrug resistance in certain cancer cells, ABCG2 is now known to be expressed in normal epithelial cells from tissues such as placenta, intestine, liver, and kidney (Maliepaard et al., 2001; Fetsch et al., 2006). Since ABCG2 is predominately localized to the apical side of biological barriers (e.g. intestine and blood brain barrier), it is considered a *protective* efflux transporter. For example, Abcg2/Bcrp1-null mice exhibit increased systemic exposure to dietary carcinogens (*e.g.* aflatoxin B1) (van Herwaarden et al., 2006), phototoxin (*e.g.* pheophorbide a) (Jonker et al., 2002), drugs (*e.g.* topotecan) and phytoestrogens (*e.g.* daidzein) (Enokizono et al., 2007) compared to wild-type mice. These mice also demonstrate higher fetal-to-maternal plasma concentration ratio for genistein and topotecan (Enokizono et al., 2007).

ABCG2 is dramatically upregulated in the mammary gland during lactation where it contributes not only to accumulation of riboflavin (vitamin B2) and other vitamins in milk, but also, for reasons unknown, to the excretion of drugs and toxins into breast milk (Jonker et al., 2005). However, the mechanism of the massive upward surge in mammary ABCG2 expression during lactation is poorly understood. Given the roles of ABCG2 in both therapeutic failure of cancer treatment and host protection from toxins, mechanistic understanding of this lactation-related phenomenon may inform a novel therapeutic strategy for ABCG2-mediated drug resistance in cancer, and provide insight into an approach for facilitated detoxification.

A wide range of modulators of ABCG2 expression have been identified, which include but are not limited to hormones (Ee et al., 2004; Wang et al., 2008a), cytokines (Evseenko et al., 2007; Le Vee et al., 2009), xenobiotics (Jigorel et al., 2006), and epigenetic factors (To et al., 2006; Turner et al., 2006). Among these, the DNA binding elements of the estrogen receptor (Ee et al., 2004), hypoxia inducible factor 1-α (Krishnamurthy et al., 2004), progesterone receptor (Wang et al., 2008a), aryl hydrocarbon

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receptor (Tan et al., 2010), peroxisome proliferator-activated receptor γ (Szatmari et al., 2006), NF-E2-related factor 2 (Singh et al., 2010), and nuclear factor-κB (Pradhan et al., 2010) have been identified in the 5' flanking region of the human *ABCG2* gene. However, none of these transcription factors has been implicated in the up-regulation of mammary gland ABCG2 during lactation, implying an existence of a novel regulatory pathway.

Lactogenesis requires the interplay between many different hormones but of primary importance is prolactin which is essential for both mammary gland development and lactation (Neville et al., 2002). Binding of prolactin to cell-surface prolactin receptor (PRLR) triggers an intracellular signalling cascade that involves Janus kinase 2 (JAK2) autophosphorylation and activation (Chilton and Hewetson, 2005; Clevenger et al., 2009). Once activated, JAK2 phosphorylates tyrosine residues on the PRLR that serves as docking sites for other proteins such as signal transducer and activator of transcription-5 (STAT5). Upon recruitment to the PRLR, STAT5 is phosphorylated by JAK2, after which it dimerizes and enters the nucleus where it binds DNA motifs called Interferon Gamma Activated Sequence (GAS) elements with the consensus sequence 5'-TTCNNNGAA-3' (Chilton and Hewetson, 2005). Examples of genes regulated in this manner include the cytokine inducible SH2 containing protein (*CISH*) (Verdier et al., 1998), and milk proteins β-casein (*CSN2*) (Gouilleux et al., 1994) and whey acidic protein (*WAP*) (Li and Rosen, 1995). In addition to JAK2/STAT5, prolactin also activates mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signalling (Clevenger et al., 2003). However, how these pathways modulate prolactin-induced gene expression is not well characterized.

Given the lactation-specific up-regulation of ABCG2 in the mammary gland, which corresponds to the prolactin surge during lactation, we hypothesized that the prolactin signalling pathways are involved in the regulation of ABCG2. In this study, we show that prolactin induces ABCG2 expression by activating not only JAK2/STAT5 but also MAPK and PI3K pathways.

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Materials and Methods

Reagents

T-47D, MCF-10A, and MCF-7 cells were purchased from the American Type Culture Collection (Manassas, VA). Primary human mammary epithelial cells (HMEC) were purchased from Clonetics, Lonza (Walkersville, MD). Fetal Bovine Serum (FBS), recombinant human insulin, RPMI-1640, DMEM, and phenol-red free DMEM/F12 were from Wiscent Inc. (Montreal, Quebec, Canada). DMEM/F12, phenol-red free high glucose DMEM, sodium pyruvate, recombinant human epidermal growth factor (EGF), horse serum, and GlutaMAX were from Gibco, Invitrogen (Life Technologies, Inc., Burlington, Canada). Actinomycin D and cholera toxin were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario). STAT5 inhibitor (N′-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide), PD98059, U0126, wortmannin, and LY294002 were from EMD Bioscience (San Diego, CA) and were dissolved to working concentrations in DMSO. FugeneHD and pGL4.23[luc2/minP] were purchased from Promega (Madision, WI). Short-interfering RNAs were purchased from Dharmacon, ThermoScientific (Lafayette, CO). Recombinant human prolactin (PRL) and recombinant growth hormone (GH) were obtained from Dr. A. F. Parlow at the NIDDK's National Hormone and Peptide Program, Harbor-UCLA Medical Center (Torrance, CA), stored as lyophilized powder, and dissolved in PBS to working concentrations. All other chemicals were purchased from Sigma.

Cell Culture and serum-starvation

T-47D cells were maintained in RPMI-1640 supplemented with 10% FBS. For serum-starvation, T-47D cells were washed twice with phenol-red free RPMI-1640, and then incubated with serum-free and phenol red-free RPMI-1640 supplemented with 0.05% fatty-acid free BSA and 0.01mg/mL holo-transferrin. MCF-7 cells were grown in high glucose DMEM containing sodium pyruvate supplemented with 10% FBS. MCF-7 cells were starved in phenol red-free DMEM supplemented with 1mM sodium pyruvate, 4mM GlutaMAX, 0.05% fatty-acid free BSA, and 0.01mg/mL holo-transferrin. MCF-10A cells were

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maintained in DMEM/F12 supplemented with 20ng/mL EGF, 0.5µg/mL hydrocortisone, 100ng/mL cholera toxin, 10µg/mL insulin, and 5% horse serum. For serum-starvation, MCF-10A cells were washed twice and incubated with phenol-red free DMEM/F12. Prior to treatment, all cells were serum-starved overnight for 18-20h. HMEC were grown as per instructions provided by Clonetics, Lonza (Walkersville, MD). In brief, HMEC were grown in MEBM supplemented with 0.4% bovine pituitary extract, 10ng/mL EGF, 5µg/mL insulin, 0.5µg/mL hydrocortisone, 15ng/mL amphotericin B and 30µg/mL Gentamicin and used in the second passage after thawing (approximately 6^{th} division). HMEC were serum-starved in phenol red-free MEBM or phenol-red-free MEBM supplemented with 5µg/mL insulin and 0.5µg/mL hydrocortisone. All cells were maintained in a humidified incubator at 37°C under a 5% CO_2 atmosphere.

RNA isolation and real-time PCR

RNA was isolated using RNeasy Kit (QIAGEN Inc. Toronto, Canada), and quantified using a Nanodrop 2000. RNA integrity was assessed by agarose gel electrophoresis. RNA was reverse transcribed to cDNA using MMLV-reverse transcriptase and random primers (Invitrogen, Life Technologies Inc., Burlington, Canada). Real-time RT-PCR was performed using inventoried Taqman primers (Applied Biosystems, Life Technologies, Burlington, Canada) and the Applied Biosystems 7500 Real-time PCR system. Relative mRNA expression was quantified using the $2^{-\Delta\Delta CT}$ method, normalizing to GAPDH mRNA expression.

Preparation of crude membrane fraction and whole cell lysate

Crude membrane fractions were prepared as described previously (Wang et al., 2003) with modifications. Each T225 flask of T-47D cells was washed twice with ice cold PBS and cells detached with a rubber policeman in ice-cold PBS containing 1mM PMSF. The cell suspension was pelleted by centrifugation at 150*g* for 5min at 4°C, the cell pellet was resuspended in hypotonic lysis buffer (10mM KCl, 1.5mM MgCl2, 10mM Tris-HCl, 1mM EDTA, 1mM PMSF, 1X protease inhibitor cocktail, pH 7.4) and incubated on ice for 10min. Cells were homogenized with a Dounce Homogenizer, and removal of

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plasma membrane was confirmed by visual inspection under a microscope. The homogenate was centrifuged at 1500*g* for 10min at 4°C, and the resulting supernatant was collected and centrifuged at 100, 000*g* for 60min at 4°C. The crude membrane pellet was resuspended in 200µL resuspension buffer (10mM Tris-HCl, 0.25M Sucrose, 150mM NaCl, 1mM PMSF, 1X protease inhibitor cocktail, pH 7.4), passed through a 26-gauge needle 10 times, and aliquoted. Aliquots were stored at -80°C. For preparation of whole cell lysate from T-47D, MCF7, MCF10A, and HMEC, cells were washed twice with ice-cold PBS (containing 1mM Na_3VO_4 and 2.5mM NaF if lysate is to be used for detection of phosphorylated proteins) and lysed with RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 2mM Na_3VO_4, 5mM NaF, 2mM EDTA, 1mM PMSF, and 1X protease inhibitor cocktail. Cells were scraped with a rubber policeman, transferred to a microcentrifuge tube and rotated end-over-end at 4°C for 20min. Cellular debris was pelleted by centrifugation at 10000rpm for 10min at 4°C. The supernatant was collected, aliquoted, and stored at -80°C. Protein concentration was measured using the Bradford Assay.

Immunodetection of protein by gel electrophoresis/western blot

Crude membrane preparations (30µg) and whole cell lysates (10µg) were resolved in 4-12% Bis-Tris gels and transferred to nitrocellulose membrane using the Novex NuPAGE SDS-PAGE system (Invitrogen, Life Technologies Inc., Burlington, Canada). Membranes were blocked overnight at 4°C prior to incubation with primary antibody. In general, blots were incubated with primary antibody overnight at 4°C and then with horseradish peroxidase conjugated secondary antibody for 1h at room temperature. Bands were visualized by enhanced chemiluminescence (ECL, GE Healthcare Life Sciences) followed by film exposure. Blots were stripped with Restore Western Blot stripping buffer (ThermoScientific) at room temperature for 20minutes and re-blocked overnight before incubating with a different primary antibody. Antibodies used were as follows: BCRP (1:200 BXP-21, Millipore), β-actin (1:20000, Sigma), JAK2 (1:1000, D2E12, Cell Signaling), phospho-STAT5 (1:4000, 71-6900, Invitrogen) STAT5A (1:2000, sc-1081, Santa Cruz), STAT5B (1:1000, sc-1656, Santa Cruz), phospho-ERK1/2 (1:1000, 197G2, Cell

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Signaling), total ERK1/2 (1:2000, L34F12, Cell Signaling), phospho-AKT (1:4000, C31E5E, Cell Signaling), and total (pan) AKT (1:10000, C67E7, Cell Signaling).

Short-interfering RNA

T-47D cells were seeded at 500, 000 cells per well on 6 well plates, grown for 24h, and transfected with siRNA using Dharmafect 1 as per manufacturer protocol (Dharmacon, ThermoScientific, Lafayette, CO). For simultaneous knockdown of both isoforms STAT5A and STAT5B, cells were transfected with 25nM custom siRNA duplex sense 5'-CUACAGUCCUGGUGUGAGAUU-3' and antisense 5'-UCUCACACCAGGACUGUAGUU-3' used previously by others (Gutzman et al., 2007). A non-targeting siRNA was used as a control (siGENOME non-targeting RNA #3, Dharmacon). For knockdown of JAK2, cells were transfected with 20nM each of two predesigned siRNA (J-003146-12 and J-003146-13) against JAK2, and for control, 20nM each of non-target siRNA 1 and siRNA 2 (ON-TARGETplus siRNA, Dharmacon). Twenty four hours after transfection, cells were incubated with fresh growth medium, grown for an additional 24h, then serum-starved overnight. Cells were either lysed to obtain whole cell lysate for assessing knockdown of protein expression by western blot, or treated with PBS (0.1%v/v) or PRL for 6h to assess mRNA expression.

Small molecule inhibitors of STAT5, MAPK and PI3K signalling

T-47D cells were serum-starved overnight and pre-treated with STAT5 inhibitor (200μM), MEK inhibitors PD98059 (20μM) and U0126 (10μM), or PI3K inhibitors LY294002 (10μM) and Wortmannin (25nM), and appropriate concentration of DMSO vehicle control for 1h. After 1h, cells were treated with PBS (0.1%) or 100ng/mL PRL for 15min to 6h.

Plasmid constructs.

The -1285/+362 ABCG2 construct reportedly previous (Bailey-Dell et al., 2001), which contains the ABCG2 proximal GAS element, surrounding 5'-flanking region and promoter, inserted into the pGL3-

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basic construct (Promega) is referred here as pGL3-ABCG2. Fragments of DNA containing the *ABCG2* distal GAS element were prepared by either annealing chemically synthesized oligonucleotides containing *Mlu*I sticky ends and corresponding to -4476/-4442*ABCG2* [forward: 5'-CGCGTCCAAATGAATATTTTCTCTGAACCCTAAGATAGCCA-3' and reverse: 5'-CGCGTGGCTATCTTAGGGTTCAGAGAAAATATTCATTTGGA-3', GAS element underlined], or by PCR amplification of the -4565/-4414*ABCG2* region using human genomic DNA and primers that contain *Mlu*I restriction sites [forward primer 5'-TGATACTAACGCGTTAAGGGACCTGACACTACCAATAAC-3'; reverse primer 5'-TGATACTAACGCGTGGAAGGTGAGAGAAAGGAATATAGC-3']. Annealed oligos and *Mlu*I digested PCR product was subsequently cloned into the pGL3-ABCG2 construct. To introduce a single mutation into the proximal GAS element of the pGL3-ABCG2 construct, site directed mutagenesis was performed using *Pfu* Turbo as per manufacturer instructions (Stratagene). Primers used to generate the ABCG2/GASmut construct were as follow: forward 5'-CATCCACTTTCTCAGCATCCCATTCACCAG-3'; reverse 5'-CTGGTGAATGGGATGCTGAGAAAGTGGATG-3'; with site of mutation underlined. Successful mutagenesis was confirmed by sequencing and the mutated insert was released by digestion with *Mlu*I/*Bgl*II and re-cloned back into the pGL3-basic vector. The pGL4-CISH construct contains the -1304 to +1 region of the human *CISH* gene cloned into the pGL4.10 vector (Fang et al., 2008). Tandem GAS constructs were cloned using chemically synthesized oligonucleotides that contain variable numbers of copies of the putative proximal GAS element (-448 to -422 region) of the human *ABCG2* gene. For GAS1v2.1, annealed oligos with sequence 5'-CAGCATCCACTTTCTCAGAATCCCATTCA-3' and 5'-GATCTGAATGGGATTCTGAGAAAGTGGATGCTGGTAC-3' were cloned into *Kpn*I/*Bgl*II digested pGL4.23[Luc2/minP] construct (Promega). GAS3v2.5 was constructed by inserting *Hind*III digested annealed oligos with sequence 5'-CAGACAAGCTTAGCATCCACTTTCTCAGAATCCCATTCAGACAGCATCCACTTTCTCAGAATCCCATTCAGACAGCATCCACTTTCTCAGAATCCCATTCAAGCTTAGACA-3' and 5'-

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GATCTGTCTAAGCTTGAATGGGATTCTGAGAAAGTGGATGCTGTCTGAATGGGATTCTGAGA AAGTGGATGCTGTCTGAATGGGATTCTGAGAAAGTGGATGCTAAGCTTGTCTGGTAC-3' into pGL4.23. GAS6v2.4 was constructed by ligating undigested annealed oligos used to construct GAS3v2.5, which contain *Kpn*I/*Bgl*II sticky ends, to GAS3v2.5. The same approach was used to construct GAS6v2mut.8 using annealed oligos 5'-CAGACAAGCTTAGCATCCACT*GG*CTCAGAATCCCATTCAGACAGCATCCACT*GG*CTCAGAAT CCCATTCAGACAGCATCCACT*GG*CTCAGAATCCCATTCAAGCTTAGACA-3'and 5'-GATCTGTCTAAGCTTGAATGGGATTCTGAG*CC*AGTGGATGCTGTCTGAATGGGATTCTGAG*C C*AGTGGATGCTGTCTGAATGGGATTCTGAG*CC*AGTGGATGCTAAGCTTGTCTGGTAC-3'. GAS sequence underlined. The pCMV-wtStat5a construct contains the rat Stat5a cloned into the pCMV-Tag 3B expression vector (Fang et al., 2009). Mouse Stat5a and Stat5b expression plasmids (pcDNA3-Stat5a and pcDNA3-Stat5b) were generous gifts from Dr. Dwayne L. Barber (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada).

Plasmid constructs were sequenced (The Centre for Applied Genomics, SickKids, Toronto, ON) and prepared using HiSpeed Plasmid Midi and Maxi Kit (Qiagen) for transfection.

Transient transfection and luciferase assay

For experiments using *firefly* luciferase reporter constructs driven by the *ABCG2* and *CISH* promoter, T-47D cells were seeded at 3 x 10^5cells/well on 12-well plates and grown for 24h. Cells were then transfected in growth medium with 500ng/well pGL3-basic, variations of the pGL3-ABCG2 constructs, or 75ng/well pGL4.10 or pGL4-CISH using 3:1 ratio Fugene HD: DNA (Promega). Twenty four hours after transfection, cells were serum-starved overnight and treated with 100ng-500ng/mL recombinant human PRL for 24h. For experiments using the tandem GAS constructs, T-47D cells were transfected as above with slight modifications. T-47D cells were seeded at 1.25 x 10^5cells on 24-well plates and transfected with 300ng/well pGL4.23 or variations of this constructs containing different copies of the proximal *ABCG2* GAS element. To overexpress rat Stat5a, cells were co-transfected with 300ng/well

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pCMV-wtSTAT5a or its empty vector for control. At the end of treatment, cells were lysed with 100µL (24well plates) or 250µL (12well plates) phosphate lysis buffer, and 10µL of lysate was used to measure luciferase activity using the Dual Luciferase Reporter Assay Kit from Promega on a Sirius Luminometer (Berthold Detection System, Pforzheim, Germany) . *Firefly* luciferase reporter activity was normalized to *renilla* luciferase activity from a co-transfected pRL-TK plasmid (5ng/well and 10ng/well for 24 and 12 well plates, respectively) and presented as fold change over vehicle and empty vector control. All experiments were conducted in triplicate wells at least three times.

Chromatin Immunoprecipitation

T-47D cells were grown to 80-90% confluence in 10cm-petri dishes, serum-starved overnight for 18-20h, and treated with PBS (0.1%v/v), indicated concentration of PRL, or 200ng/mL GH for up to 6h. For study using T-47D cells transfected with siSTAT5A/B or siRNA control, cells were transfected as per protocol for 6-well plates but scaled up to account for the larger surface area. For STAT5 inhibitor study, cells were pre-treated with 200µM STAT5 inhibitor or vehicle control (0.2% DMSO v/v) for 1h prior to prolactin treatment. Cells were fixed with 1% formaldehyde at room temperature for 10min. The cross-linking reaction was quenched with 125mM glycine and rotated at room temperature for 5min. Fixed cells were scraped in PBS containing 1mM PMSF and 1X protease inhibitor cocktail, and pelleted at 10000rpm, for 3min at 4°C. The cell pellet was frozen in an ethanol-dry ice bath and stored at -80°C until use. To immunoprecipitate STAT5, cell pellets were resuspended in 400µL TSEI (20mM Tris, 150mM NaCl, 2mM EDTA, 1% TritonX-100, 0.1% SDS, pH8.0) and sonicated at 30% amplitude, 10 pulses for 10s, twice, and cycled 5 times to a combined sonication of 100s using a Branson 450 sonifier. Insoluble debris was pelleted at 13200rpm, at 4°C for 10min, and the supernatant was pre-cleared with 10µL 50% slurry protein-A Agarose beads (Sigma). A small volume (4.25µL) was saved to quantify total input. The pre-cleared lysate (85µL) was then incubated with 2µg anti-STAT5 antibody (N-20, sc-836x, Santa Cruz) or non-specific rabbit IgG (Sigma) rotating at 4°C overnight. Immunocomplexes were precipitated with 20µL pre-blocked 50% Protein-A agarose beads/salmon sperm DNA for 1.5h at 4°C, and

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subsequently washed for 5 minutes three times with TSEI, once each with TSEII (20mM Tris, 500mM NaCl, 2mM EDTA, 1% TritonX-100, 0.1% SDS, pH8.0) and LiCl buffer (20mM Tris, 250mM LiCl, 1mM EDTA, 1% NP-40, 1% sodium deoxycholate, pH8.0), and twice with TE buffer as previously described (Ahmed et al., 2009). Immunocomplexes were eluted with 1% SDS in TE buffer at room temperature for 30min and cross-links were reversed overnight at 65°C. DNA was purified using EZ-10 Spin Column (BioBasic Inc.) and eluted with 50µL ddH2O. For quantification of STAT5 binding, real-time PCR was performed using 1µL eluted DNA in a 10µL reaction containing SsoFAST EvaGreen Supermix (BioRAD) and 100nM each of forward and reverse primer. Primers used were as follow. CISH GAS elements: forward 5'-CCCCTCTGGGTAGCTTCAG-3'; reverse 5'-CCCTGAGCAGTGAAAGGAAA-3'. ABCG2 proximal GAS element: forward 5'-AAGTTTCTCCCCTTTCCTTCC-3'; reverse 5'-ACAGGTTGCCCAGTCACAAG-3'. ABCG2 distal GAS element: forward 5'-GCTTCCTAAGGGACCTGACAC-3'; reverse 5'-GGAAGGTGAGAGAAAGGAATATAGC-3'. Recruitment was calculated as % of total input.

Statistical Analysis

All experiments, except for HMEC experiments, were conducted at least 3 times. Data presented are means ± standard error. Test of significance was performed using the student's t-test, or analysis of variance (ANOVA) followed by post-hoc pairwise multiple comparisons using the Tukey's or Dunnett's test. A p-value <0.05 was considered significant, and shown as a nominal value without correcting for multiple testing. All statistical analyses were performed using SPSS Statistics version 19 (International Business Machines Corp.).

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Results

Prolactin induces ABCG2 in T-47D cells

To examine if prolactin regulates ABCG2 expression, we first treated serum-starved PRLR-expressing T-47D human breast cancer epithelial cells with varying concentrations of prolactin for 24h. Prolactin dose-dependently increased ABCG2 mRNA and the mRNA of cytokine-inducible SH2 containing protein (CISH), a known prolactin-responsive gene (Fig. 1A). ABCG2 mRNA was maximally induced at 6h whereas CISH mRNA was induced as early as 2h after prolactin treatment (Fig. 1B). This induction was blocked by co-treatment with the transcription inhibitor actinomycin D (Supplemental Fig. 1). In addition, immunoblotting for ABCG2 protein in crude membrane preparations from T-47D cells treated with prolactin for 24h showed that ABCG2 protein is also induced in a dose-dependent manner (Fig. 1C). In contrast, other human breast epithelial cells (MCF-7, MCF-10A and primary human mammary epithelial cells, Supplemental Fig. 2 and 3), which express significantly less PRLR (Supplemental Fig. 4) did not show prolactin-induced expression of ABCG2 and CISH mRNA despite responsiveness to the aryl hydrocarbon receptor agonist TCDD, a known inducer of ABCG2, CYP1A1, and TiPARP (Tan et al., 2010; Kress and Greenlee, 1997; Ma et al., 2001).

JAK2- and STAT5-dependency in the induction of ABCG2 by prolactin

JAK2, one of the most proximal proteins in the prolactin signalling pathway, plays an important role in phosphorylating and hence modulating downstream signalling cascades. To determine the role of JAK2 in the induction of ABCG2 by prolactin, we used a combination of two siRNAs that target JAK2 to knockdown JAK2 expression in T-47D cells (Fig. 2A). JAK2-targeting siRNAs significantly attenuated the response to prolactin treatment (Fig. 2B). STAT5 represents the major effector of activated JAK2. Using a similar approach to determine if STAT5 mediates prolactin-induced expression of ABCG2, T-47D cells were transfected with a siRNA (siSTAT5A/B) that targets both isoform STAT5A and STAT5B (Fig. 3A). The prolactin induction of ABCG2 and CISH mRNA was significantly reduced in

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siSTAT5A/B-transfected T-47D (Fig. 3B). Consistent with these results, co-treatment with a novel STAT5 inhibitor (Müller et al., 2008) that partially reduced prolactin-stimulated STAT5 phosphorylation/activation (Supplemental Fig. 5) significantly blunted the induction of ABCG2 and CISH mRNA by prolactin (Fig. 3C). These experiments demonstrated that the JAK2/STAT5 pathway was important in the induction of ABCG2 by prolactin.

STAT5 recruitment to a putative proximal GAS element in the human ABCG2 gene

In silico examination of the 10kbp 5'-flanking region of the human *ABCG2* gene for GAS elements with sequence 5'-TTCNNNGAA-3' revealed a distal site at -4459 and a proximal site at -434 (Fig. 4A). ChIP analyses further showed that STAT5 was recruited to the proximal GAS element in a time-dependent manner with peak recruitment achieved at 1h after PRL treatment, but the distal GAS motif did not show significant STAT5 binding (Fig. 4B). This time-course of STAT5 recruitment was consistent with that observed for the *CISH* gene suggesting a similar mechanism of regulation. Further, STAT5 recruitment to the proximal *ABCG2* GAS element and *CISH* GAS elements was attenuated by knockdown of STAT5 expression (Fig. 4C) or by inhibition of STAT5 activation (Fig. 4D).

Evidence for a functional proximal GAS element in the ABCG2 gene

To test if the putative GAS elements were functional, we transfected T-47D with the luciferase reporter construct pGL3-ABCG2 (Bailey-Dell et al., 2001). This construct, which contained the proximal GAS element and surrounding region, was induced approximately 2 fold by prolactin (Fig. 5A). We confirmed prolactin-responsiveness of our transfection protocol using the pGL4-CISH reporter construct (Fang et al., 2008). The addition of the distal GAS element and the neighbouring region to pGL3-ABCG2 did not further enhance reporter activity (Fig. 5B), which was consistent with the ChIP data (Fig. 4B). In contrast, by introducing a single mutation into the proximal GAS element, reporter activity was significantly reduced (Fig. 5C). Similar results were observed when a different single mutation was introduced into the proximal GAS element (data not shown). To investigate whether this proximal GAS element was

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functional in isolation, various numbers of copies of the GAS element were cloned into the minimum promoter-driven pGL4.23 luciferase reporter construct (Fig. 6A). The reporter activity of these constructs was induced by prolactin in a dose- and copy number- dependent manner (Fig. 6B). In particular, GAS6v2.4 containing six tandem repeats of the proximal GAS element was very sensitive to prolactin treatment. Prolactin-induced reporter activity was synergistically increased by co-transfection with rat Stat5a (Fig. 6C), or mouse Stat5a and Stat5b (data not shown) expression vector. As evidence that this reporter activity was dependent on an intact GAS element, double mutation to each of the GAS elements, as shown using the GAS6v2mut.8 construct, completely abolished reporter activity (Fig. 6C).

Prolactin-induced ABCG2 expression is attenuated by MAPK and PI3K pathway inhibitors.

In addition to JAK2/STAT5, the MAPK and PI3K pathways are also activated by prolactin signalling. The effect of these pathways on the induction of ABCG2 by prolactin was examined using MAPK pathway inhibitors U0126 and PD98059, and PI3K pathway inhibitors LY294002 and wortmannin. These inhibitors were used at concentrations that consistently blocked activation of the MAPK or PI3K pathway as assessed by reduction of prolactin stimulated phosphorylation of ERK1/2 or AKT as shown by western blot (Fig 7A-D). Inhibition of MAPK and PI3K signalling attenuated the inductive effect of prolactin on ABCG2 but not CISH mRNA at 6h after treatment (Fig. 7E-H).

STAT5 recruitment to the proximal GAS element is not affected by MAPK and PI3K inhibitors

One potential mechanism by which small molecule-mediated inhibition of MAPK and PI3K pathways can attenuate the induction of ABCG2 by prolactin is by repressing STAT5 recruitment. To test this, we performed ChIP to assess the effect of U0126, PD98059, LY294002 and wortmannin on prolactin stimulated STAT5 recruitment. Co-treatment with these inhibitors did not affect STAT5 recruitment to the *ABCG2* proximal GAS element and the *CISH* GAS elements (Fig. 8A and 8B). This suggests that MAPK and PI3K pathways modulate prolactin-induced ABCG2 expression by a mechanism distinct from STAT5 recruitment.

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Growth hormone induces ABCG2 mRNA and STAT5 recruitment to the ABCG2 proximal GAS element. Growth hormone (GH) is a member of the peptide hormone family to which prolactin belongs and can activate similar signalling networks including JAK2/STAT5. For this reason, to examine the generalization that JAK2/STAT5 activators are potential inducers of ABCG2, T-47D cells were treated with different concentrations of recombinant human GH for 6h. The mRNA expression of ABCG2 and CISH was upregulated by GH in a dose-dependent manner (Fig. 9A). To determine if GH, like prolactin, stimulates STAT5 recruitment to the ABCG2 proximal GAS element, we performed ChIP at 1h after GH treatment (200ng/mL). GH treatment significantly increased STAT5 occupancy at the *ABCG2* proximal GAS element (Fig. 9B), demonstrating a potential role for STAT5 in GH-induced *ABCG2* expression.

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Discussion

The multidrug resistance transporter ABCG2 is upregulated in the mammary gland during lactation (Jonker et al., 2005). To date, few studies have addressed the potential regulatory mechanisms that may explain this phenomenon. In the present study, we show that prolactin upregulates ABCG2 in T-47D human breast cancer epithelial cells via activation of JAK2/STAT5, MAPK and PI3K signalling. We further identify a functional STAT5 binding sequence (GAS element) at position -434bp upstream of the human *ABCG2* transcription start site. To our knowledge, this is the first evidence of JAK2/STAT5 mediated regulation of ABCG2.

Our results extend the findings of Wang *et al*., 2008b, to provide mechanistic insight into the regulation of ABCG2 by prolactin at the transcriptional level. We used T-47D cells as a PRLR-expressing model system since the prolactin response monitored by known target genes was not observed in other mammary cell lines such as MCF-7, MCF-10A, and primary HMEC, probably due to low PRLR expression. The expression of the functional long form of the PRLR in the mammary gland of rat and mouse is temporally regulated, showing lactation-associated upregulation (Camarillo et al., 2001; Mizoguchi et al., 1996). Although experimental data to confirm this in humans is lacking, T-47D cells serve as a model system to study prolactin signalling in the human mammary gland.

Our *in silico* search for GAS elements with sequences 5'-TTCNNNGAA-3' in the proximal 10kbp 5'flanking region of the human *ABCG2* gene revealed two putative sites (Fig. 4A). We then identified the proximal GAS element (position -434) to be functional and able to recruit STAT5 upon prolactin stimulation (Fig. 4 to 6). This GAS element may serve as an important site for crosstalk between STAT5 and other nuclear receptors that bind the *ABCG2* gene. The inability for the distal GAS site to bind STAT5 agrees with the more stringent consensus STAT5 (5'-TTCC/TNG/AGAA-3') binding motif that was identified by *in vitro* DNA binding site selection using pools of double stranded oligonucleotides (Soldaini et al., 2000).

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The JAK2/STAT5 pathway is the best characterized system activated in prolactin signalling. It mediates many of the effects of prolactin such as induction of milk protein expression during lactation. Here we show that knockdown or pharmacological inhibition of JAK2 (Fig. 2) or STAT5 (Fig. 3) attenuates prolactin-induced ABCG2 mRNA expression. This demonstrates that the induction of ABCG2 by prolactin depends on the JAK2/STAT5 pathway. This has broad implications because this pathway is not only activated by prolactin but by a variety of hormones and cytokines including erythropoietin, interleukins 2, 3, 5, and 7, granulocyte-macrophage-colony stimulating factor, and growth hormone (Tan and Nevalainen, 2008). In this study, we show that growth hormone also induces ABCG2 and stimulates the recruitment of STAT5 to the proximal GAS element (Fig. 9). Since the action of growth hormone can be mediated by both the growth hormone receptor and prolactin receptor (Goffin et al., 1996; Xu et al., 2011), these results must be interpreted with caution. Nonetheless, our results suggest that JAK2/STAT5 activators are potential inducers of ABCG2.

Meyer zu Schwabedissen *et al.*, 2006, showed that the tyrosine kinase inhibitor AG1478 and MEK inhibitor PD98059 abolished epidermal growth factor (EGF)-induced ABCG2 expression. This led the authors to conclude that EGF induced ABCG2 by activation of the MAPK cascade. In the present study, we show that co-treatment with two different MEK inhibitors U0126 and PD98059 attenuates the induction of ABCG2 by prolactin. This further supports the involvement of the MAPK pathway in ABCG2 gene regulation.

Unlike the JAK2/STAT5 and MAPK pathway, regulation of ABCG2 by PI3K/AKT signalling is comparatively well studied; however, its exact role is a matter of debate. First reported by Mogi *et al*., 2003, bone marrow from Akt1 knockout mice shows significantly reduced fraction of BCRP-high expressing “side population” (SP) cells compared to wild-type mice. Furthermore, the SP fraction in bone marrow cells can be reduced by inhibiting PI3K or conversely, increased by overexpressing constitutively active AKT. Using immunofluorescence microscopy, the authors concluded that this is a result of PI3K/AKT-induced alteration of ABCG2 membrane localization but not gene expression. Similarly, using porcine kidney epithelial cell line LLC-PK1 stably expressing human ABCG2, Takada *et*

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al., 2005, showed that ABCG2 expressed in the plasma membrane was internalized by treatment with PI3K inhibitors or by overexpression of dominant-negative AKT. More recently, it was reported that neurospheres deficient in PTEN, a protein that antagonizes the action of PI3K, contained more SP cells compared to controls and PI3K inhibition effectively reduced the SP fraction, with a shift in ABCG2 localization from membrane to cytoplasm, with no changes in protein and mRNA expression (Bleau et al., 2009). Wang *et al*., 2010, using the human hepatocarcinoma PLC cell line, demonstrated that overexpression of OCT4 induces phosphorylation of AKT and the expression of ABCG2, the effect of which was blocked by inhibiting PI3K. Our findings that PI3K inhibitors reduce prolactin-induced ABCG2 expression provide further evidence of the involvement of the PI3K pathway in the transcriptional regulation of ABCG2. Together, these data support a major role for PI3K activation in ABCG2 membrane localization, and a modulatory role in ABCG2 expression in the presence of other pathways such as those activated by OCT4 or PRL.

In our study, prolactin-stimulated STAT5 recruitment to the *ABCG2* proximal GAS element was not affected by MAPK and PI3K pathway inhibitors (Fig. 8) suggesting that STAT5 recruitment to the GAS element is independent of MAPK and PI3K activation. Given that MAPK or PI3K pathway inhibitors attenuate ABCG2 induction by prolactin, we further postulate that prolactin induction of ABCG2 depends on activation of MAPK and PI3K pathways. One hypothesis is that STAT5 recruits co-regulators that are activated by MAPK and PI3K pathways, which is necessary for optimal ABCG2 gene expression (Fig. 10). The identity of these putative effector proteins remains to be determined. Intriguingly, in addition to JAK2/STAT5 that mediates many of the effects of PRL, MAPK and PI3K pathway components are over-represented in the transcriptome of the lactating mammary gland (Lemay et al., 2007; Maningat et al., 2009). All three pathways may therefore contribute to ABCG2 expression in the mammary epithelium during lactation.

It should be noted that while CISH expression was used as an indicator of prolactin responsiveness, our results suggest that there are differences in transcriptional regulation between *CISH* and *ABCG2*. JAK2 and STAT5 knockdown caused a significant induction of basal ABCG2 but not

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CISH mRNA (Fig. 2B and 3B), the effect of which was not observed with the STAT5 inhibitor (Fig. 3C). We speculate that prolonged suppression of JAK2/STAT5 signalling achieved only with siRNA may have disinhibited pathways that are negatively regulated by JAK2/STAT5, and that are positive regulators of *ABCG2* but not *CISH*. One notable example is the AHR pathway which is a positive regulator of ABCG2 (Tan et al., 2010). For reasons unknown, the basal expression of AHR-target gene CYP1A1 was induced by 50% after JAK2 knockdown (unpublished observations). The lack of effect of MAPK and PI3K pathway inhibitors on prolactin-induced CISH expression further highlights differences in transcriptional control between the two genes. The CISH gene appears to be regulated mainly by JAK2/STAT5, whereas regulation of ABCG2 is more multi-factorial.

The significance of our findings extends beyond understanding how ABCG2 is regulated by prolactin during lactation. The prolactin receptor is overexpressed in 60-95% of human breast cancers (Reynolds et al., 1997; Ormandy et al., 1997; Touraine et al., 1998; Gill et al., 2001). This is attributed to reduced phosphorylation of Ser349 within the phospho-degron of the PRLR, resulting in impaired degradation of the PRLR in human breast cancers (Li et al., 2006). Prolactin is also produced by the mammary epithelium (Reynolds et al., 1997, Clevenger et al., 1995) and has been shown to be upregulated in breast cancers compared to normal/hyperplastic epithelium (McHale et al., 2008). There is strong evidence that PRL plays an important role in breast cancer progression and for this reason, there have been efforts to find chemotherapies that target the PRL pathway (Goffin et al., 2005; Clevenger et al., 2008). In addition to its role in the biology of breast cancer progression, there is evidence that PRL can modulate the cytotoxicity of chemotherapeutic agents. Prolactin antagonists have been shown to enhance the cytotoxic effect of cisplatin, paclitaxel, and doxorubicin in breast cancer cell lines (Ramamoorthy et al., 2001; Howell et al., 2008) whereas pre-treatment with PRL can attenuate the cytotoxicity of chemotherapeutic agents taxol, vinblastine, doxorubicin, and cisplatin (LaPensee et al., 2009). Our results suggest that prolactin may confer resistance to chemotherapeutics such as doxorubicin by induction of ABCG2. The significance of this induction will require further study.

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In summary, we show that prolactin induces ABCG2 expression in T-47D human breast cancer cells. Our results are the first to demonstrate regulation of ABCG2 by JAK2/STAT5 and further support the involvement of the MAPK and PI3K pathways in the transcriptional regulation of ABCG2. These findings suggest a potential role for prolactin in the regulation of ABCG2 during lactation and offers additional mechanistic insight into the regulation of ABCG2 expression in certain breast cancer cells.

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Authorship contribution

Participated in research design: Wu, Riddick, Matthews, Harper, and Ito

Conducted experiments: Wu, Dalvi, Lu, and Yang,

Contributed new reagents or analytic tools: Matthews, Ross, and Clevenger

Performed data analysis: Wu, Harper, and Ito

Wrote or contributed to the writing of the manuscript: Wu, Riddick, Matthews, Ross, Clevenger, Harper and Ito

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Footnotes

Grants and Fellowships

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Meeting abstracts where work was previously presented

This work was presented previously, in part, at the follow meetings: Wu A, Dalvi P, Yang M, Lu X, Harper PA, and Ito S (2012) Prolactin induces BCRP/ABCG2 expression in T-47D breast cancer cells via activation of multiple signalling cascades. Experimental Biology, April 21-25, 2012, San Diego, CA. American Society for Pharmacology and Experimental Therapeutics, Bethesda, MD, USA. FASEB J 26:1047.2.; and Wu A, Dalvi P, Lu X, Yang M, Riddick DS, Matthews J, Clevenger CV, Ross DD, Harper PA, and Ito S (2012) Identification of a prolactin-responsive STAT5 binding element in the 5'-flanking region of the human BCRP/ABCG2 gene. 19th International Symposium on Microsomes and Drug Oxidations and 12th European ISSX meeting, June 17-21, 2012, Noordwijk aan Zee, Netherlands. International Society for the Study of Xenobiotics, Washington, DC, USA.

Reprint requests

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Figure Legends

Figure 1. Prolactin induces ABCG2/BCRP mRNA and protein expression in T-47D breast cancer cells. A. T-47D cells were treated with indicated concentrations of recombinant human prolactin (PRL) for 24h. Relative mRNA expression compared to vehicle control (PBS 0.1%v/v) was quantified by real-time RT-PCR. B. T-47D cells were treated with 500ng/mL PRL for the times indicated. Relative mRNA expression compared to time-matched vehicle control (0.1% PBS) was quantified by real-time RT-PCR. C. T-47D cells were treated with indicated concentrations of PRL for 24h. Crude membrane preparations were resolved by SDS-PAGE and immunoblotted for ABCG2/BCRP and β-actin. Results shown are means ± SEM (n = 3).

Figure 2. Knockdown of JAK2 attenuates prolactin-induced ABCG2/BCRP mRNA expression. T47D cells transfected with non-targeting siRNA (siCtrl) or siRNA targeting JAK2 (siJAK2) were either (A) lysed to assess JAK2 protein expression or (B) treated with PBS (0.1%v/v, vehicle) or PRL (recombinant human prolactin, ng/mL) for 6h to assess mRNA expression by real-time RT-PCR. Results are presented as fold change from siCtrl+PBS, mean ± SEM (n = 3). A mean fold change of ABCG2 or CISH under each treatment was compared between siCtrl and siJAK2 using Student's t-test (two-tailed). ABCG2: PBS, $p = 0.002$; 100ng/mL PRL, $p = 0.015$; and 500ng/mL PRL, $p < 0.001$. CISH: PBS, $p = 0.001$; 100 ng/mL PRL, $p < 0.001$; and 500 ng/mL PRL, $p < 0.001$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as compared to corresponding siCtrl treatment group.

Figure 3. Knockdown or pharmacological inhibition of STAT5A/B reduces the effect of prolactin on ABCG2/BCRP expression. T-47D cells transfected with non-targeting siRNA (siCtrl) or siRNA targeting STAT5A and STAT5B (siSTAT5A/B) were either (A) lysed to assess STAT5 protein expression by SDS-PAGE/western blot or (B) treated with PBS (0.1%v/v) or 100 ng/mL recombinant human prolactin (PRL) for 6h to assess transcript expression using real-time RT-PCR. B. Transcript

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expression is presented as fold change to siCtrl+PBS group, mean ± SEM (n = 3). A mean fold change of ABCG2 or CISH mRNA levels after PRL treatment was compared between siCtrl and siSTAT5A/B using Student's t-test (two-tailed). ABCG2: $p < 0.001$. CISH: PRL, $p < 0.001$. *** $p < 0.001$. C. T-47D cells were pretreated with DMSO (0.2%v/v) or 200μM STAT5 inhibitor for 1h, and then treated with PBS (0.1%v/v) or 100ng/mL PRL for 1h or 6h to assess transcript expression. Relative mRNA expression was quantified by real-time RT-PCR, and results shown are fold change relative to the DMSO+PBS group at each time point, mean ± SEM (n = 3). A mean fold change of ABCG2 or CISH expression after PRL treatment was compared to that in the absence of the inhibitor at each time point by Student's t-test (two-tailed). ABCG2: 1h, $p = 0.389$; and 6h: $p = 0.001$. CISH: 1h, $p = 0.007$; and 6h: $p = 0.03$. * $p < 0.05$, **$p<0.01$, Student's t-test.

Figure 4. Prolactin induces STAT5 recruitment to the 5'flanking region of the human ABCG2 gene containing a putative GAS element. A. Schematic representation of the 5'flanking region of the human ABCG2 and CISH gene and regions amplified (flanked by arrows) using real-time PCR after chromatin immunoprecipitation (ChIP). B. T-47D cells were starved overnight and treated with 100 ng/mL recombinant human prolactin (PRL) for the times indicated. STAT5 recruitment to the regions of interest was analyzed by ChIP. C. T-47D cells transfected with 25nM non-targeting siRNA (siCtrl) or siRNA targeting STAT5A and STAT5B (siSTAT5A/B) were treated with PBS (0.1%v/v) or 100 ng/mL recombinant human prolactin (PRL) for 1h. STAT5 recruitment was assessed by ChIP, and expressed as % input, mean ± SEM (n=3). Means of STAT5 recruitment values were compared between PRL-treated siCtrl and siSTAT5A/B by Student's t-test (two-tailed). ABCG2: $p = 0.003$. CISH: $p = 0.001$. No significant difference was observed with IgG. ** $p < 0.01$, Student's t-test. D. T-47D cells were pre-treated with DMSO (0.2%v/v) or 200μM STAT5 inhibitor for 1h prior to treatment with PBS (0.1%v/v) or 100ng/mL recombinant human prolactin (PRL) for 1h. STAT5 recruitment was assessed by ChIP and presented as % input, mean ± SEM (n=3). Means of STAT5 recruitment values were compared between

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PRL-treated DMSO and STAT5 inhibitor by Student's t-test (two-tailed). ABCG2: $p = 0.002$. CISH: $p = 0.055$. No significant difference was observed with IgG. ** $p < 0.01$, n.s., not significant, Student's t-test

Figure 5. Prolactin induces *ABCG2* promoter-driven luciferase reporter activity in T-47D cells. T-47D cells transfected with *firefly* luciferase reporter constructs and a *renilla* luciferase control construct were treated with PBS (0.1%v/v) or indicated concentration of recombinant human prolactin (PRL). A. T-47D cells were transfected with luciferase reporter constructs pGL3-ABCG2 or pGL4-CISH. ** $p < 0.01$, *** $p < 0.001$, One-way ANOVA followed by Dunnett's post-hoc pairwise comparison to vehicle control. B. T-47D cells were transfected with pGL3-ABCG2, or constructs containing the distal GAS element in a fragment (-4565/-4414 or -4476/-4442) of the human ABCG2 gene ligated to pGL3-ABCG2. Cells were treated with PBS (0.1%v/v) or 100 ng/mL PRL. One-way ANOVA and Dunnett's post-hoc pairwise comparison to pGL3-ABCG2 was not significant. n.s. not significant. C. T-47D cells were transfected with pGL3-ABCG2 or ABCG2/GASmut that contains a single mutation (bold italics) to the conserved/consensus putative proximal GAS element and treated with PBS (0.1%v/v) or 100 ng/mL PRL. * $p < 0.05$, Student's t-test. A to D. Data presented are fold change over PBS and empty vector control (pGL3-basic for ABCG2 constructs, or pGL4 for pGL4-CISH), normalized to *renilla* luciferase activity, and expressed as means ± SEM (n =3).

Figure 6. Prolactin induces reporter activity in T-47D cells transfected with luciferase constructs containing tandem repeats of the proximal *ABCG2* GAS element. A. Schematic representation of the luciferase reporter constructs that contain variable numbers of copies of *ABCG2* proximal GAS element (-448/-422 fragment) inserted into the pGL4.23 construct (GAS1v2.1, GAS3v2.5, GAS6v2.4). GAS6v2mut.8 contains six tandem repeats of the mutated *ABCG2* proximal GAS element -448/-422 fragment (represented with an X in the schematic). Numbers denote position relative to nucleotide 1 in the backbone pGL4.23 construct. B. T-47D cells transiently transfected with the indicated reporter constructs were treated with the indicated concentration of recombinant human prolactin (PRL, ng/mL)

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for 24h. *Renilla*-normalized luciferase activity was expressed as fold-change over PBS treatment. Significance was tested by One-way ANOVA followed by Dunnet's post-hoc pairwise comparison to PBS treatment for each reporter. *p<0.05, **p<0.01. C. T-47D cells were transfected with GAS6v2.4 or GAS6v2mut.8 along with pCMV-Tag3B (empty vector) or a plasmid constitutively expressing wild-type rat STAT5A (pCMV-wtStat5a) and treated with PBS (0.1%v/v) or 100 ng/mL PRL for 24h. *Firefly* luciferase reporter activity was normalized to *renilla* luciferase activity from co-transfected pRL-TK plasmid, and presented as fold change over vehicle and empty expression vector control (pCMV-Tag3B+PBS). Mean fold changes in reporter activity after prolactin treatments were compared between pCMV-Tag3B and pCMV-wtStat5a using the Student's t-test (two-tailed). GAS6v2.4: p = 0.001. GAS6v2mut.8: p = 0.004 (reduction). ** p < 0.01, Student's t-test. B and C. Results shown are mean ± SEM (n = 3).

Figure 7. Pharmacological inhibition of MAPK and PI3K signalling attenuates the induction of ABCG2 by prolactin. T-47D cells were pre-treated with pathway specific inhibitors U0126 (10μM, MEK1/2 inhibitor, panel A and E), PD98059 (20μM, MEK1 inhibitor, panel B and F), LY294002 (10μM, PI3K inhibitor, panel C and G), wortmannin (WT, 25nM, PI3K inhibitor, panel D and H) or DMSO (0.1%v/v) for 1h, and subsequently treated with PBS (0.1%v/v) or 100 ng/mL recombinant human prolactin (PRL) for 15min to assess ERK1/2 phosphorylation, 30min to assess AKT phosphorylation, or 6h to assess mRNA expression. A to D. Whole cell lysates (10μg) were resolved by SDS-PAGE and immunoblotted for phosphorylated ERK1/2 (p-ERK1/2) and total ERK1/2 or phosphorylated AKT (p-AKT) and total AKT. E to G. *ABCG2* or *CISH* transcript expression was quantified by real-time RT-PCR, and *GAPDH*-normalized values were shown as fold change relative to DMSO+PBS group, mean ± SEM (n = 3-5). PRL-induced fold change was compared between DMSO and an inhibitor of the signalling pathway using Student's t-test (two tailed). U0126: ABCG2, p = 0.006; and CISH, p = 0.282. PD98059: ABCG2, p = 0.024; and CISH, p = 0.180. LY294002: ABCG2, p = 0.001; and CISH, p =

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0.184. Wortmannin: ABCG2, p = 0.007; and CISH: p = 0.272. * p<0.05, **p<0.01, n.s., not significant, Student's t-test.

Figure 8. Prolactin-induced STAT5 recruitment to the *ABCG2* proximal GAS element is not attenuated by inhibitors of MAPK and PI3K signalling. T-47D cells were pre-treated with MAPK inhibitors (panel A: 20μM PD98059 or 10μM U0126), PI3K inhibitors (panel B: 10μM LY294002 or 25nM Wortmannin, WT) or DMSO (0.1%v/v) for 1h and subsequently treated with PBS (0.1%v/v) or 100ng/mL recombinant human prolactin (PRL) for an additional 1h. STAT5 recruitment to the regions of interest was analyzed by ChIP. Data presented are recruitment as mean % input ± SEM (n = 3). Two-way ANOVA for ABCG2 or CISH showed no statistically significant difference in STAT5 recruitment between DMSO and each signalling inhibitor. No significant difference was observed for IgG. n.s. not significant compared to prolactin in DMSO (i.e., in the absence of inhibitor).

Figure 9. Growth hormone induces ABCG2 expression and STAT5 recruitment to the proximal GAS element of the *ABCG2* gene in T-47D cells. A. T-47D cells were treated with indicated concentrations of recombinant human growth hormone (GH) for 6h to assess mRNA expression. Relative mRNA expression was quantified by real-time RT-PCR and presented as fold change over untreated cells (0.1%PBS), mean ± SEM, n = 3. B. T-47D cells were treated with PBS (0.1%v/v) or 200 ng/mL GH for 1h and STAT5 recruitment to the regions of interest was assessed by ChIP. A non-specific rabbit IgG antibody was included as negative control. Recruitment is presented as % of total input, mean ± SEM (n = 4). ** p<0.01 compared to PBS treatment, Student's t-test.

Figure 10. Schematic representation of the hypothetical interaction between prolactin signal transduction pathways in the transcriptional regulation of the human *ABCG2* gene. Activation of JAK2/STAT5 by prolactin induces the recruitment of STAT5 to the proximal GAS element of the *ABCG2* gene. STAT5 recruitment alone is insufficient to induce transcription. Instead, STAT5 must

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recruit co-regulators (shown here by X and Y) that are activated by MAPK and PI3K pathways to induce transcription of the *ABCG2* gene.

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Figure 1.

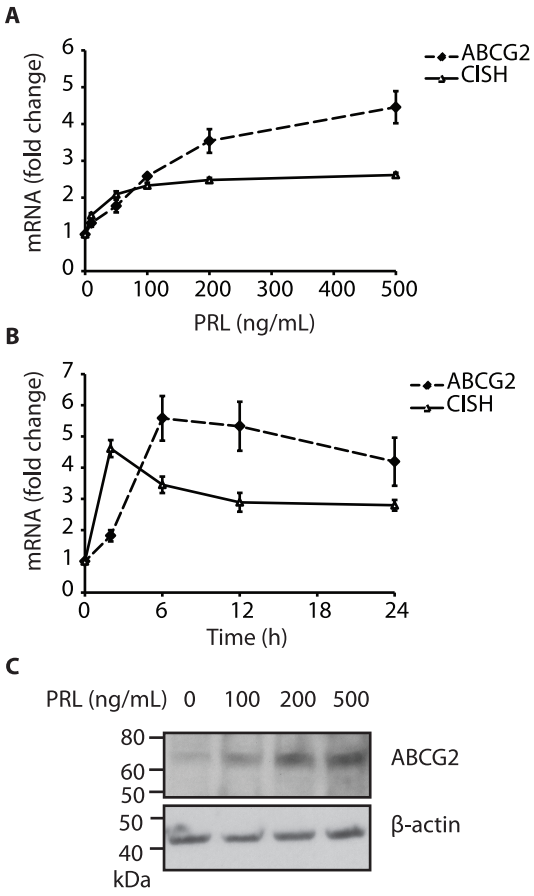

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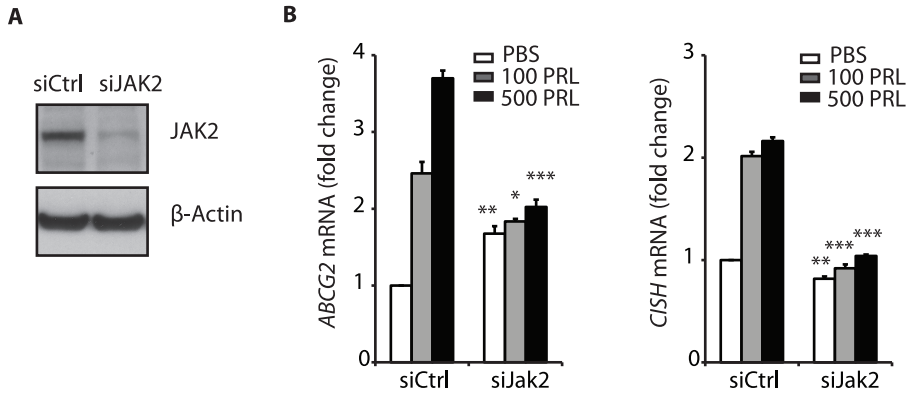
Figure 2.



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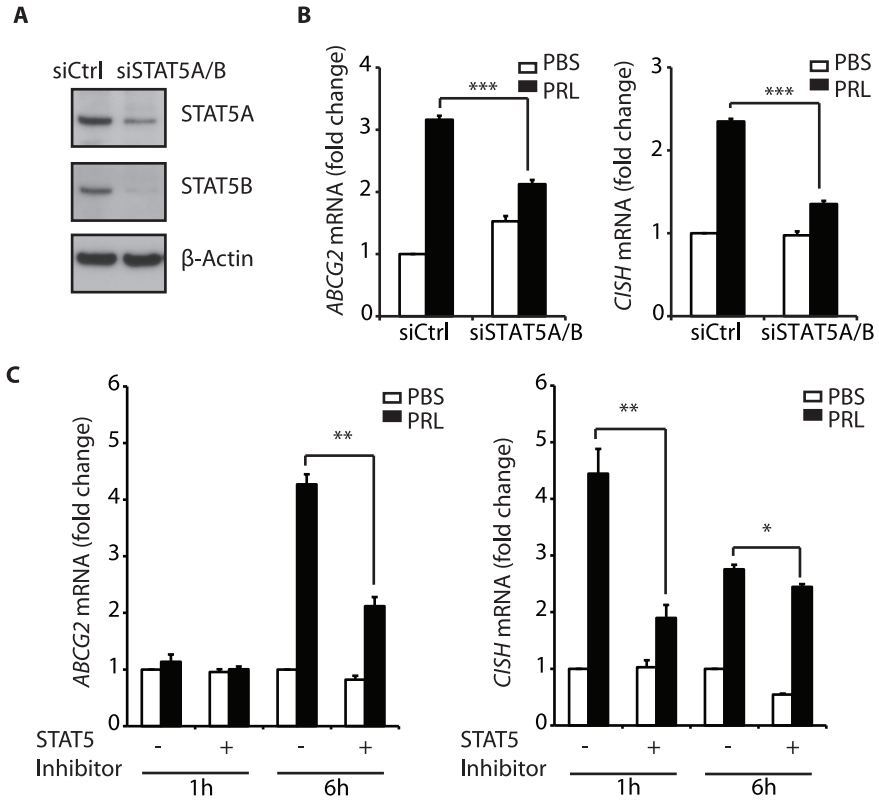

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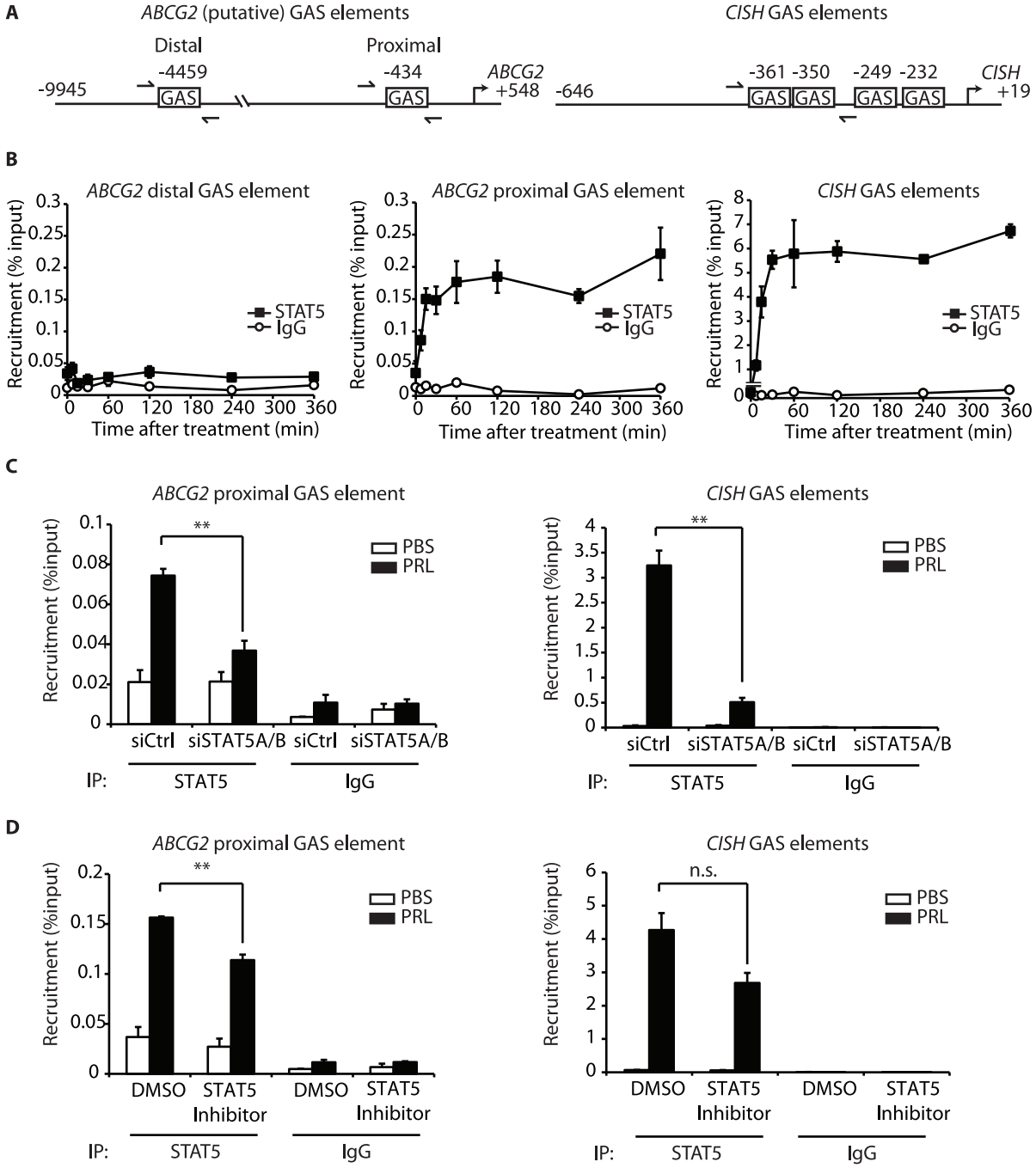

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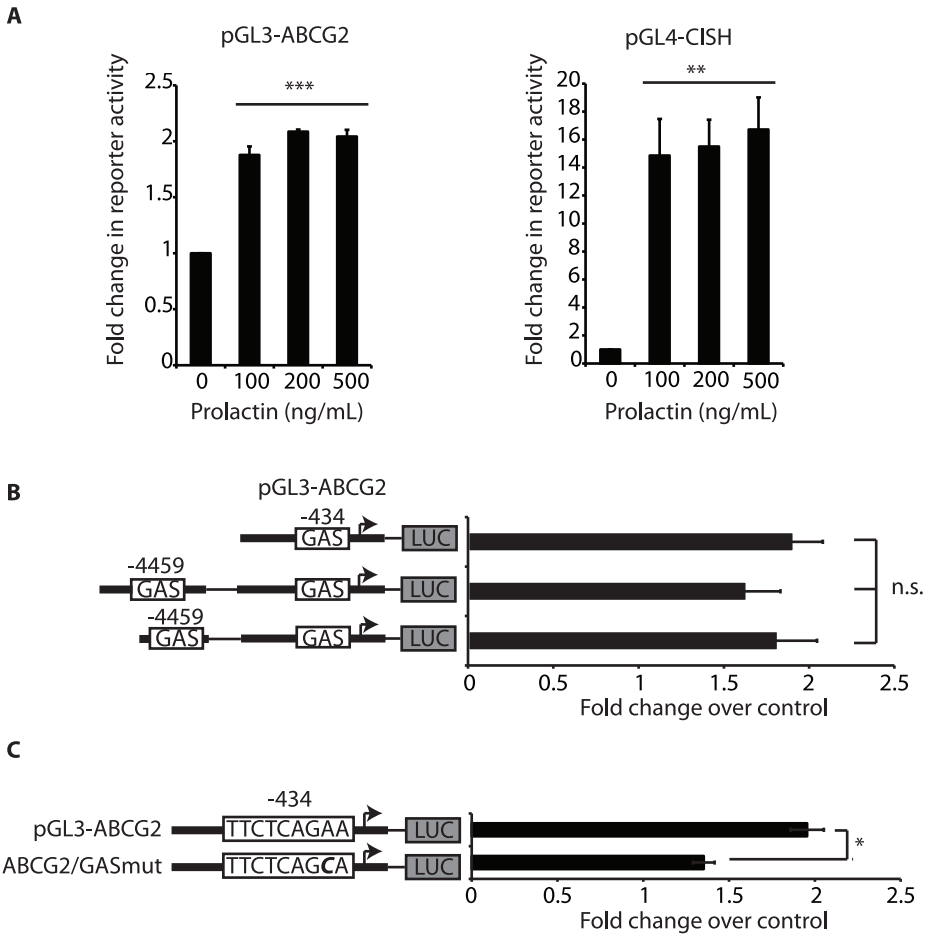

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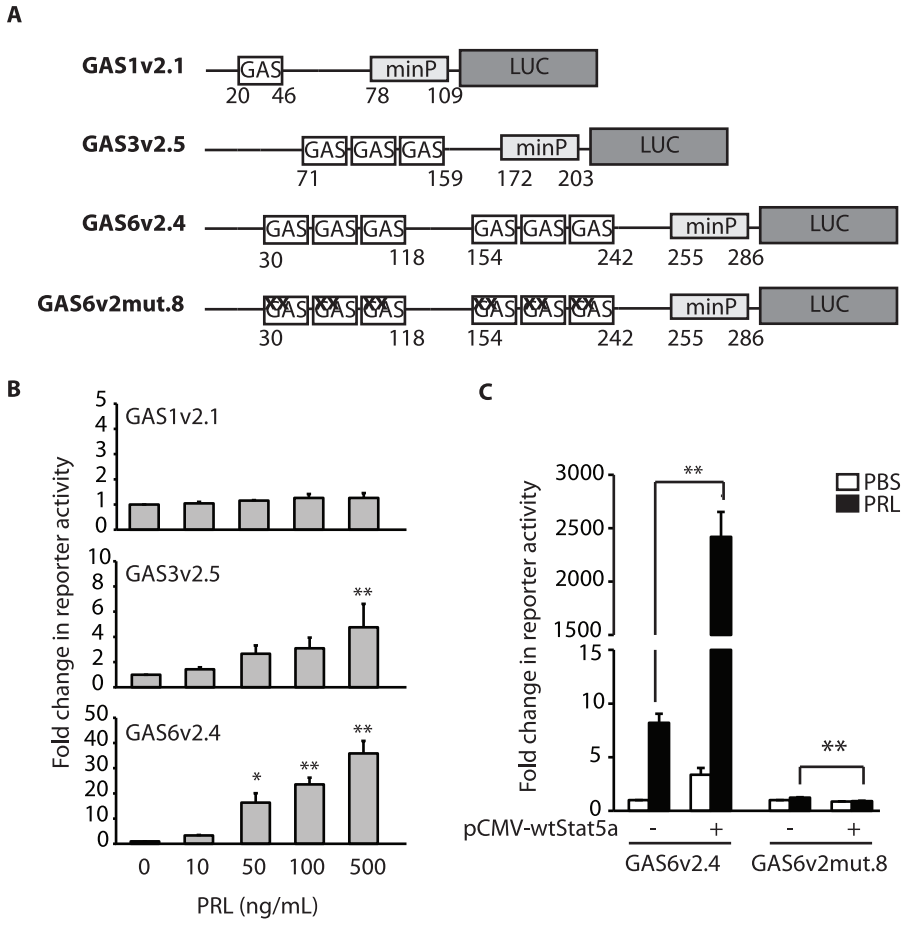

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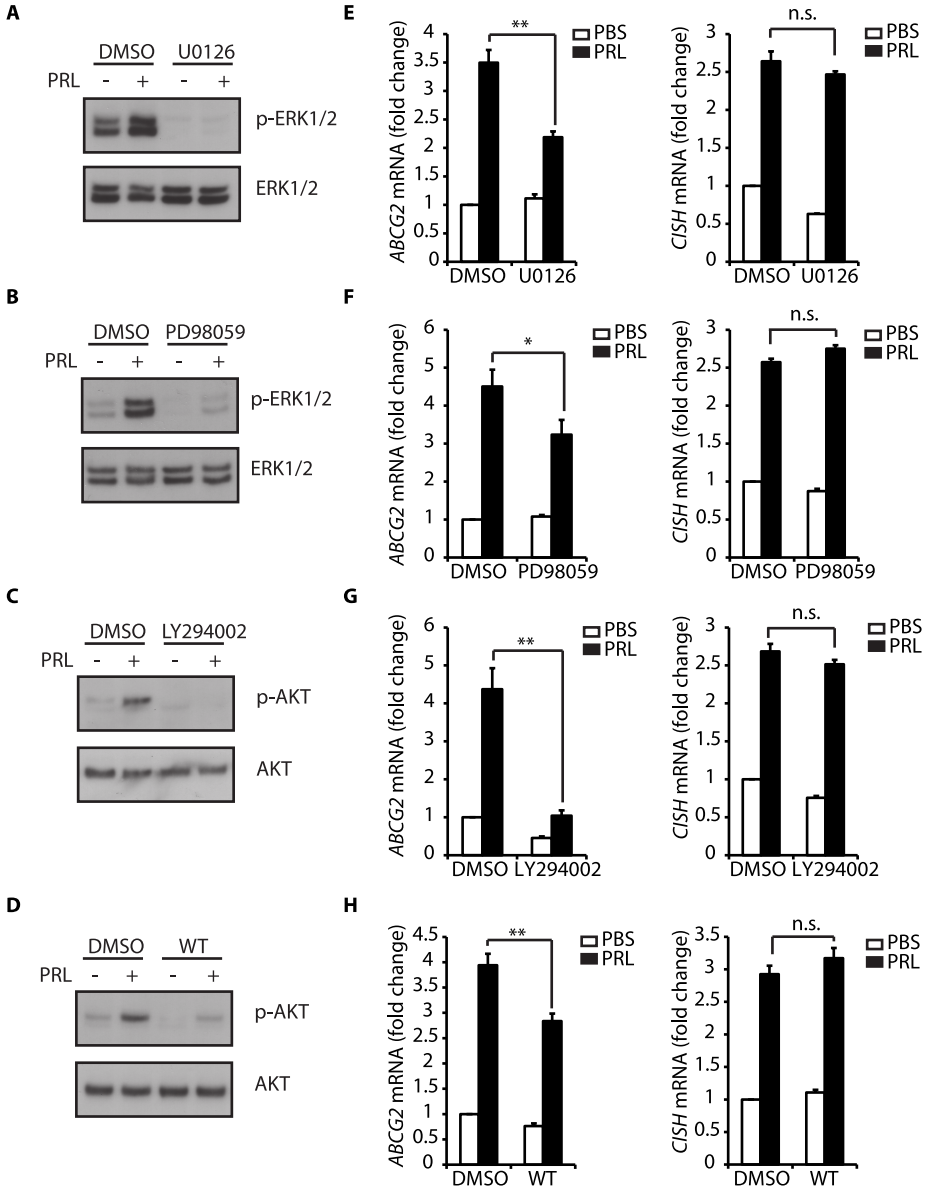

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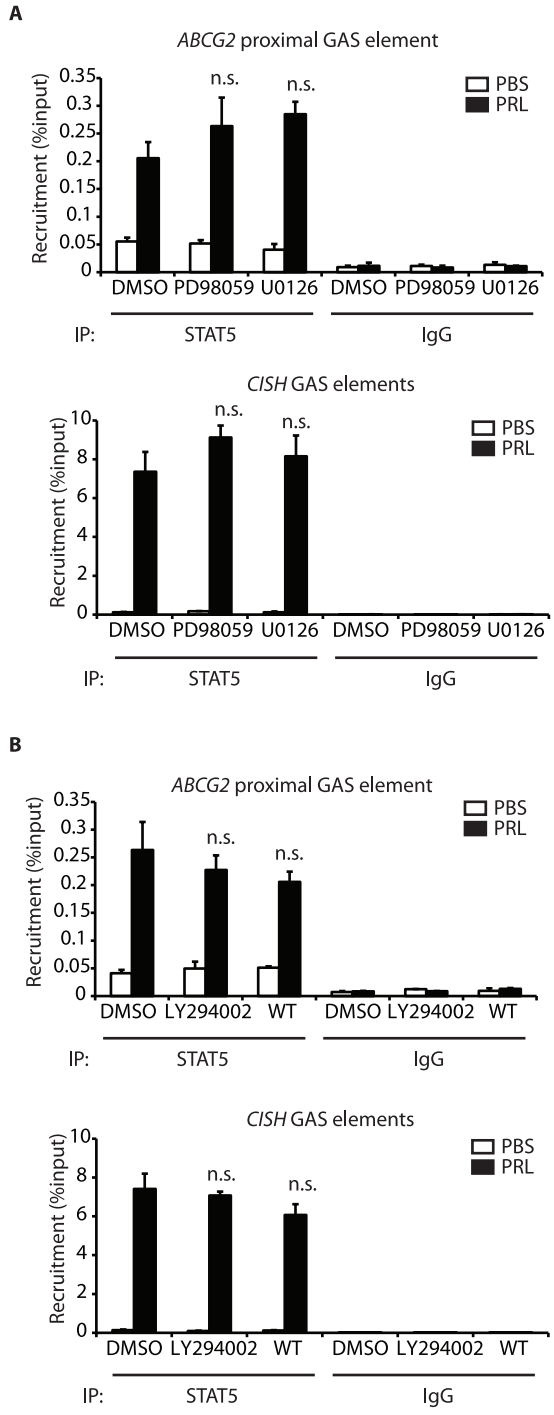

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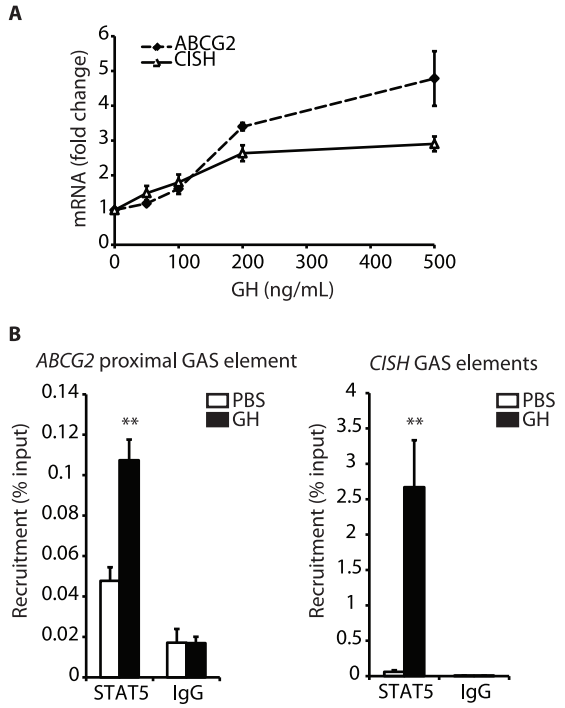

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Figure 10.