**Notch1 regulates the expression of the multidrug resistance gene ABCC1/MRP1 in cultured cancer cells**

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Edited by Susan Band Horwitz, Albert Einstein College of Medicine, Bronx, NY, and approved July 7, 2011 (received for review January 13, 2011)

**Multidrug resistance (MDR) is a barrier to successful cancer chemotherapy. Although MDR is associated with overexpression of ATP-binding cassette (ABC) membrane transporters, mechanisms behind their up-regulation are not entirely understood. The cleaved form of the Notch1 protein, intracellular Notch1 (N1IC), is involved in transcriptional regulation of genes. To test whether Notch1 is involved in the expression of multidrug resistance-associated protein 1 (ABCC1/MRP1; herein referred to as ABCC1), we measured N1IC and presenilin 1 (PSEN1), the catalytic subunit of γ-secretase required for Notch activation. We observed higher levels of N1IC and PSEN1 proteins as well as higher activity of N1IC in ABCC1-expressing MDR MCF7/VP cells compared with parental MCF7/WT cells. Reducing N1IC levels in MCF7/VP cells with either a γ-secretase inhibitor or shRNA led to reduction of ABCC1. By contrast, ectopic expression of N1IC in MCF7/WT cells led to increased expression of ABCC1 and associated drug resistance, consistent with expression of this transporter. Inhibition of ABCC1 reversed drug resistance of N1IC-overexpressing stable cells. Using an ABC1 promoter construct, we observed both its reduced transcriptional activity after blocking the generation of N1IC and its increased transcriptional activity in stable cells overexpressing N1IC. ChiP and gel-shift assays revealed an interaction between a specific promoter region of ABCC1 and the N1IC-activated transcription factor CBFI, suggesting that the regulation of ABCC1 expression by Notch1 is mediated by CBFI. Indeed, deletion or site-directed mutagenesis of these CBFI binding sites within the ABCC1 promoter region attenuated promoter-reporter activity. Overall, our results reveal a unique regulatory mechanism of ABCC1 expression.**

**The emergence of multidrug resistance (MDR) is frequently a major impediment to the successful use of cancer chemotherapy (1). MDR is defined as cross-resistance to a wide variety of structurally, chemically, and mechanistically unrelated anticancer drugs (1, 2). Explication of MDR in cancers has revealed many mechanisms (3–7), including overexpression of ATP-binding cassette (ABC) membrane transporters such as P-glycoprotein (ABCB1/MDR1; Pgp) (8, 9), MDR protein 1 (ABCC1/MPR1; herein referred to as ABCC1) (10), and breast cancer resistance protein (ABCG2/BCRP) (11). Although many cellular mechanisms are important in MDR (3–7), overexpression of ABC membrane transporters in cancers is considered to be a primary determinant of the MDR phenotype.**

Human ABCC1 transports a broad range of xenobiotics (2), physiological substrates (12), and other compounds (13, 14). Although ABCC1 is ubiquitously expressed and may play a role in normal physiology to protect tissues (10), overexpression of ABCC1 in tumors has been clearly associated with clinical drug resistance in lung and esophageal cancers, leukemia, and childhood neuroblastoma (10, 15–17). Regulation of the ABCC1 gene at its 5′ untranslated promoter region is associated with various transcription factors, such as GC elements (−91 to +103) that are essential for binding of Sp1; putative AP-1 sites (−511 to −492) interacting with a complex of c-jun and jund; and putative E-box elements (−1020 to −2008) interacting with MYC/MYCN (18–20). In addition to transcriptional regulatory factors, we and others showed that increased ABC11 expression in cancers was associated with loss of functional p53 (21–23).

**Human Notch is a heterodimeric type I transmembrane receptor protein encoded by one of four Notch genes (Notch1–Notch4) (24). Via interaction with ligands of the Delta and Jagged families, Notch generates an intracellular C-terminal fragment (NIC) that translocates into the nucleus, where it can switch the CSL/NIC/coactivator complex transactivates various target genes, including those of the HES/HEY families (25). N1IC is oncogenic in T-cell acute lymphoblastic leukemia mouse mammary tumors and human breast cancer cells (26–28); intracellular Notch1 (N1IC) has been implicated in the resistance of thymocytes to glucocorticoids (29), in melphalan and mitoxantrone resistance in myeloma and other malignant lymphoid cell lines (30), and in resistance to adriamycin, cisplatin, etoposide, and taxol in MCF7 and MOLT4 cells (31). Although the involvement of Notch1 in anticancer MDR is becoming clear, its contribution to the expression of ABC transporters has not been extensively examined. Thus, we asked here whether and how Notch1 contributes to the overexpression of ABC transporters in tumor cell lines, thereby conferring MDR.**

**Results**

**Drug-Resistant MCF7/VP Cells Express High Levels of N1IC.** Our preliminary results showed that ABCC1 expression is inversely related to the expression of truncated transmembrane Notch1 (N1) in MCF7 breast cancer cells and their etoposide-resistant subline, MCF7/VP. We confirmed this inverse relationship in MDR human leukemic CEM/VMM1-5 cells that overexpress ABCC1. However, we could not find this relationship in cells expressing either ABCG2/BCRP or ABCB1/MDR1 (Fig. S1). We therefore hypothesized that Notch1 may affect the transcriptional regulation of the ABCC1 gene.

Notch signaling involves the generation of the biologically active intracellular form of Notch1 (N1IC) from truncated transmembrane Notch1 (N1). We observed higher levels of N1IC in both MCF7/VP and CEM/VMM1-5 cells that overexpress ABCC1 but not in cells that overexpress either ABCB1 (CEM/VLB100) or ABCG2 (MCF7/MX) (Fig. 1B). We also found higher levels of Hey1, a target of N1IC (32), in both MCF7/VP and CEM/VMM1-5 cells (Fig. 1B). Moreover, MCF7/VP cells exhibited three times more CBFI promoter activity compared with MCF7/WT cells (40.0 ± 6.8 vs. 14.0 ± 5.3, respectively; mean ± SE; P < 0.05)

**Author contributions:** S.C., M.L., and W.T.B. designed research; S.C., M.L., P.-L.R.E., and L.M. analyzed data; and S.C., M.L., and W.T.B. wrote the paper.

The authors declare no conflict of interest.

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to better understand the higher enzymatic generation of N1IC seen in the MCF7/VP cells, we measured the expression of presenilin 1 (PSEN1), which inhibit processing of N1 into N1IC. We determined that the IC50 of DAPT in MCF7/VP cells is 103.4 ± 6.8 in MCF7/WT and MCF7/VP cell lines, respectively (mean ± SE; n = 3; P < 0.05 by Student’s t test). (C) Representative Western blot of PSEN1 (Upper) and densitometry analysis from three independent Western blots of PSEN1 (Lower) (mean ± 5D; n = 3; P < 0.05, Student’s t test).

**Inhibition of Notch1 Down-Regulates ABC1 Expression.** To down-regulate N1IC, first treated cells with a γ-secretase inhibitor, N-[3-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine β-butyler ester (DAPT), which inhibits processing of N1 into N1IC. We determined that the IC50 of DAPT in MCF7/VP cells is 103.4 ± 1.4 μM (Fig. 2A). We treated MCF7/VP cells with 1, 10, or 50 μM of DAPT and measured N1IC and ABCB1 proteins by immunoblotting. As expected, DAPT inhibition of γ-secretase caused a reduction of N1IC at 50 μM compared with DMSO control (Fig. 2B). Furthermore, 50 μM DAPT reduced ABCB1 levels to ~50% in these cells compared with DMSO controls after 48 h (Fig. 2B). Although these results are of interest, we note that γ-secretase inhibitors have nonspecific effects on different Notch homologs as well as on Notch1 (24), thereby making interpretation of these results complicated. Accordingly, we introduced a shRNA against Notch1 into these cells to suppress Notch1 expression. As shown in Fig. 2C, the levels of N1 and N1IC were effectively suppressed by the shRNA, and expression of ABCB1 was also reduced.

**Ectopic Expression of N1IC Induces the Expression of ABC1 and Drug Resistance.** We then asked whether ectopic expression of N1IC in drug-sensitive MCF7/WT cells can affect the expression of ABC1 and drug sensitivity. We established two clones of MCF7/WT cells stably expressing N1IC under G418 selection and found that the ectopic expression of N1IC was comparable to the N1IC levels in MCF7/VP cells and substantially higher than the levels in control cells (Fig. 3A). As expected, the higher N1IC expression in these clones was associated with higher N1IC activity (3.5-fold), as measured by CBF1 promoter activity (Fig. 3C). Both N1IC clones highly overexpressed Hey1, a direct target of Notch1 (Fig. 3B). Remarkably, ectopic expression of N1IC caused increased expression of ABC1 mRNA and protein in the MCF7/WT cells (Fig. 3A and B) to levels comparable to those seen in MCF7/VP cells (Fig. 3A). Clone ICN1-c4 was as resistant to etoposide as the MCF7/VP cells were, with an IC50 value of 15.8 ± 1.3 (Fig. 3D). By comparison, the IC50 values for MCF7/WT-pcDNA3 and MCF7/VP were 3.1 ± 1.6 and 17.3 ± 1.2, respectively (Fig. 3D). Protein levels of ABCB1/MRD1 and ABCG2/BCRP in these N1IC-overexpressing stable clones were not affected (Fig. S2). These results indicate that ectopic expression of N1IC induces expression of ABC1, which confers resistance to etoposide.

**Inhibition of ABC1 Reverses Drug Resistance of N1IC-Overexpressing Stable Cells.** To investigate whether drug resistance can be reversed by reducing ABC1 activity/levels in the presence of activated Notch1, we treated N1IC-overexpressing stable cell clones (ICN1-c4) with either the ABC1-specific inhibitor reversan (33) or ABC1 siRNA to knock down ABC1 expression and tested cells for changes in drug sensitivity. We found that the ABC1 inhibitor reversan sensitizes ICN1-c4 cells to etoposide (17.8-fold) (Fig. 3E) and knockdown of ABC1 reverses drug resistance of ICN1-c4 cells to etoposide (5.1-fold) (Fig. S3E). These results strongly support the idea that Notch1 exerts its effect on ABC1-mediated drug responsiveness in large part through its induction of ABC1.

**Notch1/CFB1 Interacts with the Promoter Region of ABC1. ChIP analysis.** Because N1IC regulates transcription in part through CFB1 (34, 35), we analyzed the ABC1 promoter for putative

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**Fig. 1.** MDR cells expressing ABC1 have higher levels of N1IC and PSEN1 compared with controls. (A) Representative Western blot of N1IC with an antibody against N1IC (activated N1). (B) RT-PCR of Hey1, a gene regulated by N1IC. (C) Luciferase assay of N1IC activity using a CBF1 promoter-luciferase construct (41). CBF1 promoter activities were 14.0 ± 5.3 and 40.0 ± 6.8 in MCF7/WT and MCF7/VP cell lines, respectively (mean ± SE; n = 3; P < 0.05 by Student’s t test). (D) Representative Western blot of PSEN1 (Upper) and densitometry analysis from three independent Western blots of PSEN1 (Lower) (mean ± 5D; n = 3; P < 0.05, Student’s t test).

**Fig. 2.** Inhibition of N1IC in MCF7/VP cells reduces expression of ABC1. (A) MTT assay to test cellular toxicity of DAPT in MCF7/VP cells. IC50 (μM) was 103.4 ± 1.4 in MCF7/VP cells. (B and C) Representative Western blots showing N1 and N1IC (mN1A) and ABC1 levels in MCF7/VP cells after treatment with DAPT (B) or shRNA against Notch1 (C).
of inhibiting ABCC1 activity, because MAML interacts with CBF1 in Notch-mediated gene transcription (39). As shown in Fig. S4, DN-MAML1 inhibits CBF1 activity by more than 50%, and, importantly, it also inhibits ABCC1-luciferase promoter constructs [full-length (−808) and minimal (−411) promoters]. We cannot expect more than this degree of inhibition because of the ubiquity of the interactions, as with CBF1 mentioned above. These data support our conclusion that N1IC activates ABCC1 expression through binding to the CBF1 element.

**Notch1 Regulates the Expression of ABCC1 at the Transcriptional Level.** To elucidate the basis of this association of N1IC with ABCC1 expression, we asked whether Notch1 regulates ABCC1 expression at the transcriptional level. To test this hypothesis, we transfected MCF7/VP cells with a full-length ABCC1 promoter-luciferase (MRP-luc) construct (p-2008) that covers the promoter region of ABCC1 from −2008 to +103 bp (relative to the transcriptional start site) and treated these cells with different concentrations of DAPT. As seen in Fig. 5A, we found that DAPT treatment at 1, 10, or 50 μM progressively inhibited transcriptional activity of the ABCC1 promoter by 87.8 ± 3.7%, 82.8 ± 7.0%, and 66.8 ± 7.8% of control, respectively. Moreover, ABCC1 promoter activity was increased in MCF7/WT cells stably expressing N1IC (21.3 ± 3.1 in ICN1-c4 vs. 5.8 ± 1.1 in controls) (Fig. 5B). To identify the ABCC1 promoter region(s) that respond to Notch1 signaling, we measured the transcriptional activities of a series of ABCC1 promoter-deletion constructs in MCF7/VP cells (Fig. 5C). These deletion constructs were derived from p-2008 and contained promoter regions spanning from nucleotide +103 to nucleotides −91 (p-91), −411 (p-411), −600 (p-600), and −1123 (p-1123). All constructs exhibited promoter activities compared with the basic vector pGL2 (Fig. 5C).

Sequence analysis of the region between bases +103 and −411 (Fig. 4A) revealed four putative CBF1 binding sites that are similar to the consensus CBF1 binding element, GTGGGAA (36). To determine whether these sites are responsible for the promoter activities, we deleted I, II, III, or IV alone, or a combination of two, or I/II/III together, or I/II/III/IV together within the p-91 construct and measured promoter activities in MCF7/ VP cells. Among different combinations, only deletion of I/II/III together led to higher activity than p-411 did (Fig. 5D), possibly because of Notch1/CBF1 enhanced binding to the fourth CBF1 binding site on the promoter region. In contrast, deleting the fourth CBF1 binding site alone or all four binding sites together within the p-411 construct (p-411-Del IV, p-411-Del I/II/III/IV) led to reduced promoter activity by 60% and 46%, respectively. Similar results were observed when we deleted the fourth CBF1 binding site within the p-91 construct (p-91-Del IV). Furthermore, when we mutated five of seven nucleotides within the fourth CBF1 binding site, the promoter activity was reduced to levels similar to those seen in the p-91-Del IV construct (Fig. 5D). Altogether, these data indicate that the fourth CBF1 binding site alone contributes to the promoter activity of ABCC1. Overall, we conclude that N1IC regulates ABCC1 expression through a transcriptional regulatory mechanism that likely involves CBF1 binding to the ABCC1 promoter.

**Discussion.** We have shown here that the developmental protein Notch1 regulates the expression of the ABC transport protein ABCC1 through CBF1 regulatory sites in its promoter. Our results suggest a unique mechanism for some forms of anticancer MDR. Notch has many targets (37), and some appear to be related to drug resistance in tumor cells. There have been several reports of the involvement of N1IC in the resistance of cells to glucocorticoids (29), melphalan and mitoxantrone (30), and adriamycin (31). We have shown herein that N1IC is involved in the transcriptional regulation of ABCC1 and likely contributes to the ABCC1-mediated resistance to etoposide. ABCC1 is an ABC transporter involved in some forms of anticancer MDR. Our analyses of the
region between bases +103 and −411 in the ABCC1 promoter revealed four sequence elements that are similar to the consensus CBF1 binding element, GTGGGAA (36). We demonstrated by EMSA and ChIP assay that the N1IC/CBF1 complex can bind directly to the putative CBF1 binding elements on the promoter of ABCC1. By decreasing the level of N1IC with either DAPT or shRNA, we observed reduced expression of ABCC1 in drug-resistant cells. Furthermore, we showed that drug-resistant cells can be sensitized by reducing ABCC1 activity/levels in the presence of activated Notch1, supporting the idea that Notch1 exerts its effect on ABCC1-mediated drug responsiveness in large part through its induction of ABCC1. In addition, drug-sensitive cells ectopically expressing N1IC demonstrated increased expression of ABCC1 and etoposide resistance. We conclude that N1IC regulates ABCC1 expression through a transcriptional regulatory mechanism involving CBF1. We note that, although CBF1 was indicated as the mediator of canonical Notch-regulated transcriptional activation (40, 41), ABCC1 has not been described previously as a canonical Notch/CBF1 target.

In Notch signaling, the intracellular form of Notch (N1C) that results from enzymatic cleavage of Notch regulates many genes involved in cellular differentiation, development, and apoptosis (25). The proteolytic generation of N1IC is an essential step in the activation of Notch signaling (34). We showed herein that the level of N1IC is increased in epipodophyllotoxin-resistant cells that are characterized by MDR through the overexpression of ABCB1/MDR1 and ABCG2/BCRP. It is clear that this Notch1 mechanism involving CBF1 is related to the increased level of PSEN1 in MDR cells remains to be determined. In the selection of MDR cells, drug-sensitive cells are continuously exposed to sublethal concentrations of an anticancer drug (43), and, as a consequence, MDR cells experience various epigenetic and genetic changes (1). Accordingly, the increased level of PSEN1 expression in our MDR cells may be a likely consequence of one of these changes.

Finally, although some studies reflect relations between ABC1 expression and clinical (multi)drug resistance (44–46), it is virtually impossible to ascribe clinical drug resistance to expression of any ABC transporter; one can only attempt to make associations. Moreover, an Oncomine (www.oncomine.org) search revealed two papers showing relationships between expression of ABC1 and Notch1 in certain cancers (35, 47), but those relationships are of necessity weak because the key measure for Notch is its activity, not necessarily its expression. These potential clinical correlations will require investigation in the future.
**Materials and Methods**

**Cells and Culture Conditions.** Cell-culture media DMEM and RPMI 1640 (Bio-Whittaker) were supplemented with 10% FBS (Gemini) and 2 mM l-glutamine (BioWhittaker). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. MCF7/WT, the etoposide-resistant subline MCF7/VP (43), and mitoxantrone-resistant MCF7/MX cells (48) were all cultured in DMEM. Human leukemic CEM cells, the teniposide-resistant CEM/VML1-S (49), and vinblastine-resistant CEM/VLB100 (50) cells were maintained in RPMI medium 1640.

**Establishment of Stable Cell Lines Expressing N1**<sup>T</sup>. MCF7 cells were seeded at 2 × 10<sup>5</sup> cells per well in 24-well plates 1 d before transfection with pICN1 (SI Materials and Methods) or pcDNA3 control plasmids by using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, cells were trypsinized, diluted 10-fold, replated, and then allowed to grow in medium containing G418 (1 mg/mL; Calbiochem) for 2008 to +103 bp (relative to the transcriptional start site) in MCF7/VP cells. Shown is the average relative promoter-luciferase activity of triplicates from two independent experiments; error bars represent the range of average values. (D) ABCC1 p-411 and p-91 promoter-luciferase reporter assay with CBF1 deletion or mutation constructs in MCF7/VP cells. Experiments were done as in C.

**Deletion and Site-Directed Mutagenesis and Luciferase Reporter Assay.** Deletion and site-directed mutagenesis was done with the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies). ABCC1 promoter construct p-411 served as a template to generate the deletion constructs—p-411–Del I, p-411–Del II, p-411–Del III, p-411–Del IV, p-411–Del I/II, p-411–Del I/III, and p-411–Del I/II/III/IV—in which one, two, three, or four putative CBF1 binding sites were deleted: I, GTGGAGA; II, GTGGGCC; III, GTGGGGG; and IV, GTGGGGC. We used ABCC1 promoter construct p-91 as the template to generate deletion construct p-91–Del IV, in which the fourth putative CBF1 binding site was deleted (IV, GTGGGGC) and mutation constructs p91-M3 (AGTGGAG), p91-M4 (AGTAGGC), and p91-M5 (AGTACGC), in which 3, 4, or 5 nt were simultaneously mutated within the fourth putative CBF1 binding site, respectively.

Luciferase reporter assays were performed as described previously (21). MCF7/VP cells were grown in 6-well plates and cotransfected with 4 µg of ABCC1 promoter-luciferase plasmid DNA and 0.4 µg of β-galactosidase reporter plasmid DNA. At 18 h later, the cells were harvested and replated at a density of 5 × 10<sup>5</sup> cells per well in 24-well plates. DAPT was added to the medium 8 h later at final concentrations of 1, 10, or 50 µM. The control cells were treated with 0.1% DMSO only. After 48 h, cell lysates were analyzed for both luciferase and β-galactosidase activities. Relative luciferase activities were normalized with β-galactosidase, and fold changes were calculated by dividing the relative luciferase activities of DAPT-treated samples by the relative luciferase activities of the control samples.

**Cytotoxicity Assay.** Cells were seeded at 3,000 cells per well in 96-well plates and incubated at 37 °C overnight. Various concentrations of etoposide (VP16; Sigma) were then added to each well and incubated for 96 h, after which the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described (51). The IC₅₀ value was calculated by using GraphPad Prism software (GraphPad Software Inc.).

**EMSA.** EMSA was performed as described in refs. 53 and 54. Nuclear extracts from MCF7/VP cells were prepared as described in ref. 54. The oligonucleotide probes for EMSA were synthesized (IDT) (see SI Materials and Methods for sequences of probes). The assignment of nonspecific versus specific binding bands was based on three comparisons (47): (i) the specific binding band should be seen from the putative CBF1 probe–protein complex as well as from the conserved CBF1 probe–protein complex; (ii) the specific binding band should be eliminated by the addition of a molar excess of unlabeled competitor DNA; and (iii) the binding of each probe to each competitor should be as strong as binding of probe to the protein.
The CBF1 binding site was amplified from PCR with the input DNA (1%) or DNA isolated from precipitated chromatin as templates and primers flanking the putative CBF1 binding sites in the AC011 promoter (see SI Materials and Methods for details).

ACKNOWLEDGMENTS. We thank the following individuals: Drs. Marsha Rosner and Jieun Yun for providing guidance in performing the ChIP assays; Martina Vaskova for administrative assistance; Dr. Kimberly E. Foreman for providing the DN-MAML1 plasmid and helpful experimental insights; and Nandita Bhandamar and Tsu-Lei Ho for their experimental assistance. This work was supported by National Institutes of Health Grants R01 CA40570 (to W.T.B.) and P01 AG025531 (to L.M.) and the University of Illinois at Chicago (W.T.B.). This investigation was conducted in a facility constructed with support from Grant C06RR15482 from the National Center for Research Resources, National Institutes of Health.


