ZNF423 Is Critically Required for Retinoic Acid-Induced Differentiation and Is a Marker of Neuroblastoma Outcome

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DOI 10.1016/j.ccr.2009.02.023

SUMMARY

Retinoids play key roles in differentiation, growth arrest, and apoptosis and are increasingly being used in the clinic for the treatment of a variety of cancers, including neuroblastoma. Here, using a large-scale RNA interference-based genetic screen, we identify *ZNF423* (also known as *Ebfaz*, *OAZ*, or *Zfp423*) as a component critically required for retinoic acid (RA)-induced differentiation. ZNF423 associates with the RARα/RXRα nuclear receptor complex and is essential for transactivation in response to retinoids. Downregulation of *ZNF423* expression by RNA interference in neuroblastoma cells results in a growth advantage and resistance to RA-induced differentiation, whereas overexpression of *ZNF423* leads to growth inhibition and enhanced differentiation. Finally, we show that low *ZNF423* expression is associated with poor disease outcome in neuroblastoma patients.

INTRODUCTION

The vitamin A metabolite retinoic acid (RA) is essential for embryonic and adult growth. It plays key roles in development, differentiation, and homeostasis. These diverse effects of RA are exerted primarily through the ability to differentially regulate gene expression mediated by the retinoic acid receptors (RARs). RAR belongs to the superfamily of nuclear hormone receptors that are ligand-regulated transcription factors (Chambon, 1996). RAR functions through heterodimerization with retinoid X receptor (RXR), which is the common partner for several other nuclear receptors, such as vitamin D receptor (VDR), peroxisome proliferator-activated receptor (PPAR), and thyroid

hormone receptor (T3R) (Mangelsdorf et al., 1995; McKenna and O'Malley, 2002). In addition, RXR can also form homodimers. As the critical dimerization component, RXR is therefore a master regulator of many hormone responses.

RAR/RXR heterodimers constitutively associate with retinoic acid response elements (RAREs) in promoters of target genes (Chambon, 1996). In the absence of ligand, RAR/RXR actively represses transcription through association with the corepressors NCoR and SMRT and recruitment of histone deacetylases (HDACs) that prevent opening of the chromatin (Chen and Evans, 1995; Horlein et al., 1995). Binding of RA to RAR induces a conformation change of the complex. Subsequently, corepressors are released and coactivator complexes are recruited to

SIGNIFICANCE

Cancer biomarkers make it possible to foretell cancer outcome (prognosis) or responses to therapy (prediction). Human neuroblastoma, the most common childhood solid tumor, has a broad range of clinical outcomes ranging from spontaneous regression to extremely aggressive disease. We show here that *ZNF423* is a prognostic biomarker for human neuroblastoma independent of *MYCN* amplification. We also establish a causal role of ZNF423 in retinoic acid (RA)-induced differentiation and proliferation of neuroblastoma cells. Thus, *ZNF423* may also predict responses to RA-based therapies in the clinic. More generally, our results underscore that the identification of components of key signaling pathways using genetic screens can yield biomarkers with clinical utility.

activate transcription. This ligand-dependent exchange of coregulators requires adaptor proteins such as TBLR1 (Perissi et al., 2004). Many of the coactivators, including CBP/p300, PCAF, and members of the p160 family (SRC1, TIF-2/GRIP1, and ACTR/RAC3/AIB1), possess histone acetylase (HAT) activity that promotes transactivation of RAR/RXR (Chen et al., 1997; Onate et al., 1995; Voegel et al., 1996). In contrast, ligand-dependent corepressors such as RIP140, LCoR, and PRAME recruit HDACs or polycomb group (PcG) proteins to ligand-bound RAR/RXR complexes to repress their activities (Cavailles et al., 1995; Epping et al., 2005; Fernandes et al., 2003).

RA signaling through RAR/RXR and the subsequent activation of target genes induce differentiation, cell-cycle arrest, and apoptosis in many cell types. Consequently, RA displays distinct anticarcinogenic activities, and it is currently in use or being tested as a therapeutic agent for several human cancers (Altucci and Gronemeyer, 2001; Freemantle et al., 2003). For example, RA is used to treat patients suffering from acute promyelocytic leukemia, in which translocations of *RARα* give rise to the *PML-RARα* chimeric gene (Grignani et al., 1998; He et al., 1998). The resulting fusion protein is a constitutive repressor that inhibits wild-type RARα activity, thereby preventing myeloblast differentiation at physiological levels of RA. Pharmacological concentrations of RA alleviate this dominant-negative block by allowing dissociation of corepressors and recruitment of coactivators to activate transcription. Furthermore, loss of expression of the RA target gene *RARβ*, an isoform of *RAR*, is involved in the progression of a diverse range of solid tumors (Altucci and Gronemeyer, 2001; Freemantle et al., 2003). As a chemoprevention, RA treatment can be used to restore *RARβ* expression. For example, restoration of *RARβ* expression by RA therapy in premalignant oral lesions is associated with a clinical response (Xu et al., 1995).

RA therapy has also been used in the clinic to treat human neuroblastoma, a childhood tumor arising in the peripheral sympathetic nervous system (Brodeur, 2003). Some tumors regress spontaneously, but the majority of neuroblastoma patients exhibit aggressive tumors with poor clinical outcome despite intense therapy. While a subset of these aggressive tumors is identified by genomic amplification of the *MYCN* proto-oncogene, less is known about additional genetic factors that control neuroblastoma tumor progression (Brodeur, 2003). High expression of the neurotrophic receptor TRKA has been identified as prognostic for favorable outcome, whereas TRKB and its ligand, the brain-derived neurotrophic factor (BDNF), are frequently expressed in unfavorable aggressive tumors with *MYCN* amplification (Brodeur, 2003; Schramm et al., 2005). In addition, the expression of FYN kinase is prognostic for good outcome of patients independent of *MYCN* amplification (Berwanger et al., 2002). Signaling through FYN kinase controls neuroblastoma cell differentiation and proliferation. RA signaling has also been implicated in human neuroblastoma, because high levels of either all-*trans* RA or 13-*cis* RA induce cell proliferation arrest and morphological differentiation of human neuroblastoma cell lines (Reynolds et al., 1994; Sidell et al., 1983). A phase III randomized trial demonstrated that treatment with 13-*cis* RA administered after completion of intensive chemoradiotherapy significantly improved event-free survival in high-risk neuroblastoma (Matthay et al., 1999). This RA therapy has now become

standard practice to treat high-risk neuroblastoma patients after bone marrow or stem cell transplantation.

Human ZNF423 and its mouse and rat homolog Zfp423 are transcription factors containing 30 Krüppel-like C_2H_2 zinc fingers (Turner and Crossley, 1999). Zfp423 was first described as a transcriptional partner of Ebf1 (Olf-1), a transcription factor implicated in olfactory epithelium and lymphocyte development in the rat (Tsai and Reed, 1997, 1998). Independently, Zfp423 was then identified as a cofactor interacting with the Smad1/Smad4 complex in bone morphogenetic protein (BMP) signaling pathways (Hata et al., 2000; Ku et al., 2006). The zinc-finger clusters of ZNF423 mediating these two signaling pathways are separable and independent from each other. In both pathways, ZNF423 assembles a transcriptional complex by binding to distinct partners and DNA sequences. Zfp423 is required for cerebellar development and plays important roles in olfactory neurogenesis and central nervous system (CNS) midline patterning in mice (Cheng and Reed, 2007; Cheng et al., 2007; Warming et al., 2006). Here we identify ZNF423 as a component of RA signaling and demonstrate that it plays a key role in RA-induced differentiation in several cell types, including neuroblastoma.

RESULTS

Zfp423 Is a Component of the RA Signaling Pathway

In response to RA, mouse F9 embryonic teratocarcinoma cells differentiate into extraembryonic endoderm-like cells, recapitulating early stages of mouse embryogenesis (Rochette-Egly and Chambon, 2001). Therefore, F9 cells have been used extensively as a model to investigate RA signaling in vitro. To identify genes involved in RA signaling, we performed a large-scale RNA interference (RNAi)-based loss-of-function genetic screen in F9 cells using a collection of 28,256 short hairpin RNA (shRNA) vectors, which target 14,128 mouse genes (Figure 1A; see also the Supplemental Experimental Procedures available online). Using retroviral infection, we introduced the entire shRNA library polyclonally into F9 cells. The infected cells were plated at low density and exposed to 1 μM all-*trans* RA (henceforth referred to as simply RA). After 4 weeks of RA selection, the resistant colonies were pooled and total genomic DNA was isolated. The shRNA vectors were recovered by PCR amplification and recloned as a pool to construct a mini library. Using this functionally selected mini library, which was enriched for shRNA vectors that can confer resistance to RA, we performed a second-round selection in F9 cells. To avoid a "passenger" effect from irrelevant shRNA vectors, low multiplicity of infection was used for infection. The resistant colonies from the second-round RA selection were individually isolated. The shRNA inserts were recovered by PCR, recloned, and subjected to DNA sequence analysis to reveal their identities as described previously (Berns et al., 2004). As an initial validation, each of the identified shRNA vectors was individually introduced into F9 cells by retroviral infection to retest its ability to confer resistance to RA. As a negative control, a functional shRNA targeting *GFP* of *Aequorea victoria* was used throughout this study.

Using this approach, we identified seven different shRNA vectors that were able to confer RA resistance in F9 cells when expressed. We found one shRNA targeting the mouse retinoid X receptor *Rxrα* (sh*Rxrα*) (Figure 1B), which validated the screen,
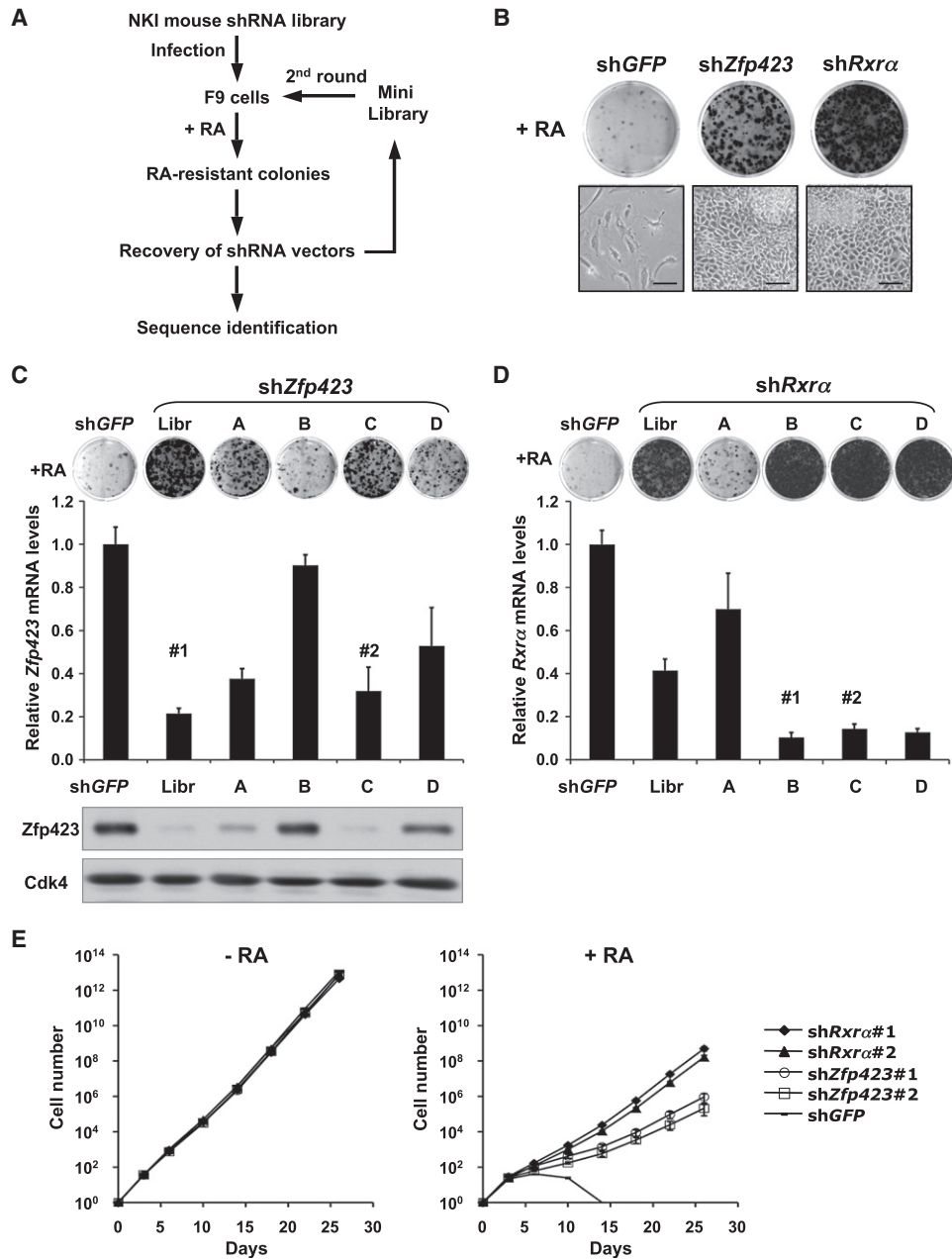
Figure 1. A Genome-wide RNAi Screen Identifies *Zfp423* as a Component of the Retinoic Acid Signaling Pathway

(A) Schematic outline of the retinoic acid (RA) resistance screen performed in mouse F9 cells. A mouse shRNA library polyclonal virus was produced to infect F9 cells. The infected cells were treated with 1 μM all-*trans* RA (hereafter referred to as simply RA) for 4 weeks, and shRNA inserts were recovered from the resistant colonies as described previously (Berns et al., 2004). A second-round screen was performed to enrich for shRNAs that confer RA resistance.

(B) Individual shRNAs from the library targeting *Zfp423* and *Rxrα* confer RA resistance. F9 cells expressing the control sh*GFP*, sh*Zfp423*, or sh*Rxrα* were treated with 1 μM RA for 3 weeks, after which the cells were photographed, fixed, and stained. Scale bars = 25 μm.

(C and D) Validation of independent shRNAs targeting *Zfp423* and *Rxrα*. The functional phenotypes of nonoverlapping shRNAs targeting each gene are indicated by the colony formation assay in 1 μM RA. The knockdown ability of each shRNA was measured by examining the mRNA levels of the intended target gene by qRT-PCR and the protein levels by western blotting. Error bars represent SD of triplicate independent experiments.

(E) RA resistance by *Zfp423* RNAi is dependent on RA signaling. Proliferation curves according to the 3T3 protocol of F9 cells expressing shRNAs targeting *Zfp423*, *Rxrα*, or *GFP* in the absence and presence of 1 μM RA are shown. Error bars represent SD of triplicate independent experiments.

since downregulation of this receptor leads to decreased transcriptional response to RA (see below). In addition, we also identified one shRNA targeting *Zfp423* (sh*Zfp423*) (Figure 1B).

To rule out that the shRNAs conferred RA resistance due to off-target effects, we designed and tested additional nonoverlapping shRNAs against each of the genes identified in the

A

B
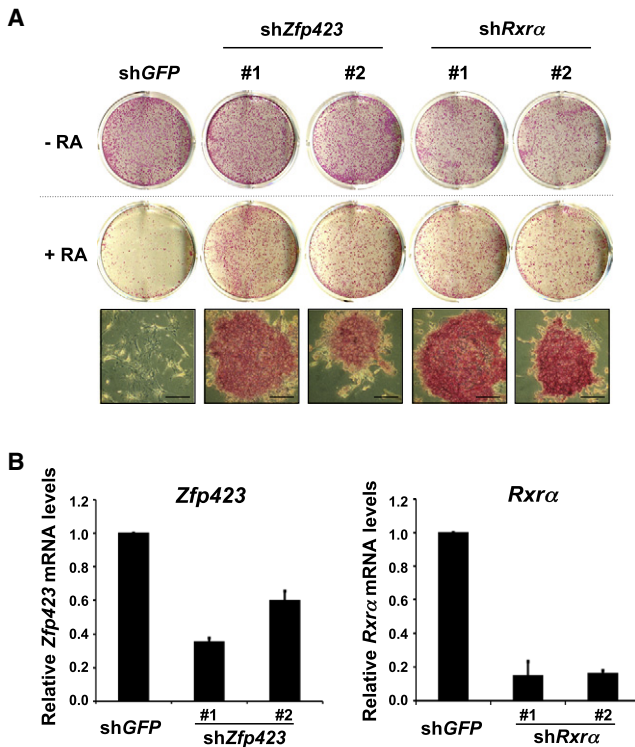
Figure 2. Zfp423 Is Also Required for RA-Induced Differentiation in Mouse Embryonic Stem Cells

(A) Downregulation of *Zfp423* by RNAi in E14T mouse embryonic stem (ES) cells confers resistance to RA-induced differentiation. E14T cells expressing shRNAs against *GFP* (control), *Zfp423*, or *Rxrα* were grown in the absence or presence of 1 μM RA for 1 week, after which cells were fixed, stained for alkaline phosphatase (AP), and photographed. Scale bars = 50 μm.

(B) mRNA levels of *Zfp423* and *Rxrα* in E14T mouse ES cells expressing shRNAs targeting *GFP*, *Zfp423*, or *Rxrα*. Error bars represent SD of triplicate measurements.

screen. We only considered a gene identified from the screen as a genuine hit if at least two nonoverlapping shRNAs were able to suppress expression of the intended target gene and confer RA resistance (Echeverri et al., 2006). We found a direct correlation between the knockdown abilities of the vectors and their ability to confer resistance to RA (Figure 1C), indicating that *Zfp423* was a genuine hit from the screen. Two of the most potent shRNAs, sh*Zfp423*#1 and sh*Zfp423*#2, were used throughout this study. We also generated multiple active shRNAs for the gene *Rxrα*, which we considered as a positive control (Figure 1D). Two of the most potent shRNAs, sh*Rxrα*#1 and sh*Rxrα*#2, were used in subsequent experiments. For the remaining five candidate genes, we did not observe a correlation between knockdown of the intended gene and the ability to confer resistance to RA when additional shRNA vectors were tested (data not shown), and thus these genes were not studied further.

Next, we examined whether the RA resistance mediated by suppression of *Zfp423* was due to the inhibition of RA signaling or to a general growth advantage unrelated to RA. We performed a long-term proliferation assay with F9 cells expressing shRNAs against *Zfp423*, *Rxrα*, or *GFP* in the absence or presence of exogenous RA using the 3T3 protocol (Figure 1E). In the absence

of RA, no significant growth difference was detected in any cell lines. When exposed to 1 μM RA, the cells expressing shRNAs targeting *Zfp423* or *Rxrα* continued to proliferate, while the control cells were drastically inhibited. These data argue against a generalized growth advantage for the *Zfp423* knockdown F9 cells and suggest a specific resistance to RA-induced growth arrest.

Zfp423 Is Also Required for RA-Induced Differentiation in Mouse Embryonic Stem Cells

In addition to F9 cells, mouse embryonic stem (ES) cells have been used as another cellular system to study RA-induced differentiation in vitro (Rohwedel et al., 1999). We observed that mouse ES cells also express a high level of Zfp423 (data not shown). Therefore, we examined the role of Zfp423 in RA-induced differentiation in this cellular system. shRNAs against *Zfp423*, *Rxrα*, or *GFP* were introduced into E14T mouse ES cells by retroviral infection. As expected, the knockdown abilities of these shRNAs in E14T cells were comparable to those seen in F9 cells (Figure 2B; Figures 1C and 1D). The infected cells were plated at low density and cultured in the absence or presence of 1 μM RA for 1 week. The surviving cells were then stained for alkaline phosphatase (AP), a marker of undifferentiated ES cells. In the absence of RA, there was no significant difference in cell growth and maintenance of the undifferentiated state in any knockdown lines (Figure 2A). In the presence of RA, proliferation of the control cells was severely inhibited and the remaining differentiated cells failed to stain for AP (Figure 2A). In contrast, cells stably expressing shRNAs against *Zfp423* or *Rxrα* continued to proliferate and stained positively for AP (Figure 2A). Hence, Zfp423 appears to be also required for RA-induced differentiation in mouse ES cells.

ZNF423 Is a Transcriptional Cofactor of Retinoic Acid Receptors

Zfp423 was first implicated as a transcriptional partner of Ebf1 in olfactory epithelium and lymphocyte development in the rat and was then identified as a transcriptional activator and Smad1/Smad4 cofactor in regulating BMP signaling. Therefore, we tested whether ZNF423 can also act as a cofactor for RARα/RXRα transactivation. ShRNAs targeting *Zfp423*, *Rxrα*, or *GFP* were cotransfected with a reporter gene containing consensus RAREs linked to luciferase (RARE-Luc) into F9 cells (Epping et al., 2005). Both shRNAs against *Zfp423* inhibited the reporter gene activation by RA, similar to the shRNAs targeting *Rxrα* (Figure 3A), indicating that Zfp423 is required for transcriptional activation of Rarα/Rxrα in response to RA. As an independent cellular system, we performed the RARE-Luc reporter assays in NTERA2 human embryonic teratocarcinoma cells, which also express a high level of ZNF423 (Figure S1). Similarly, shRNAs targeting human *ZNF423* and *RXRα* (see below) also suppressed RA-induced expression of the reporter gene (Figure 3B). Conversely, expression of both wild-type ZNF423 and FLAG-ZNF423ΔN (which lacks the first 60 amino acids but contains all 30 zinc fingers) was able to hyperactivate the RARE-Luc reporter in response to RA (Figure 3C). The difference in the ability of ZNF423 and FLAG-ZNF423ΔN to activate reporter expression probably reflects the fact that FLAG-ZNF423ΔN was more highly expressed than wild-type ZNF423
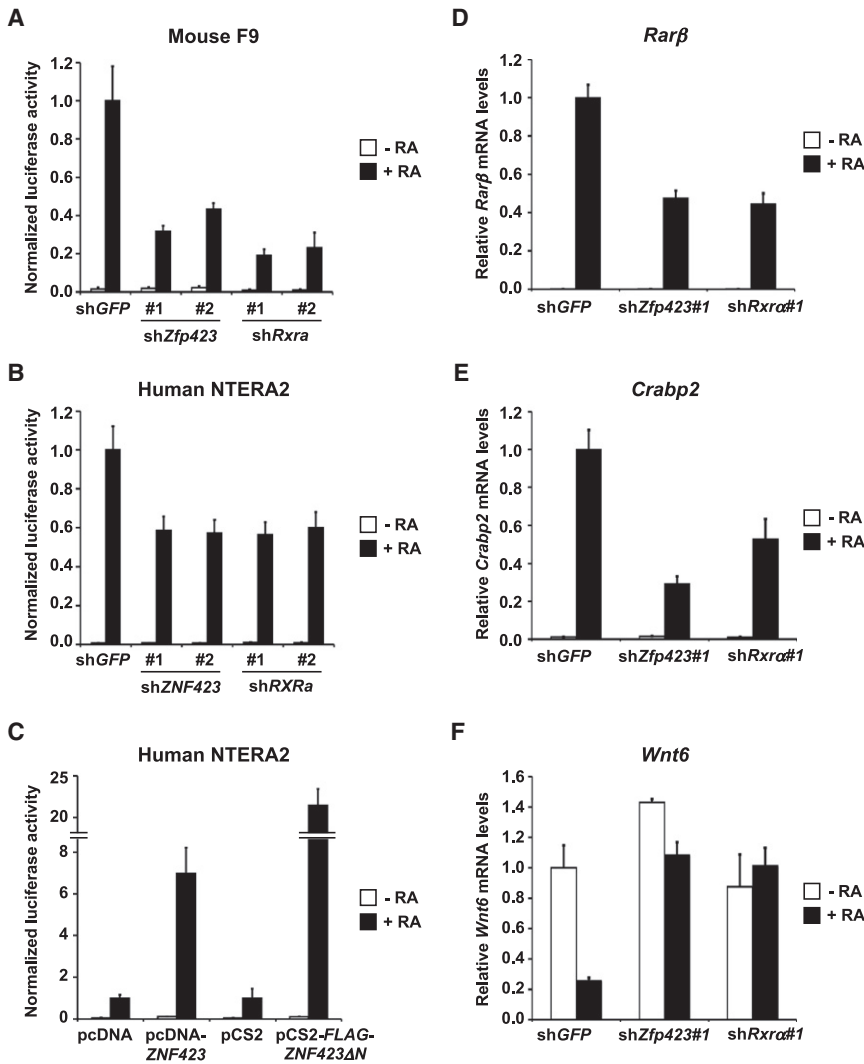
Figure 3. ZNF423 Is a Transcriptional Cofactor for RXRα/RARα

(A–C) Activation of a retinoic acid response element-luciferase (RARE-Luc) reporter gene by RXRα/RARα requires ZNF423. Normalized luciferase activities shown represent ratios between luciferase values and *Renilla* internal control values and are the average ± SD from three independent transfections.

(A) Downregulation of *Zfp423* by RNAi inhibits transcriptional induction of the RARE-Luc reporter gene in mouse F9 cells in response to 24 hr of 1 μM RA stimulation.

(B) Suppression of *ZNF423* by RNAi inhibits the activation of the RARE-Luc reporter gene in human NTERA2 cells in response to 1 μM RA treatment.

(C) Overexpression of both wild-type ZNF423 and the epitope-tagged mutant FLAG-ZNF423ΔN (which lacks the first 60 amino acids but contains all 30 zinc fingers) was able to hyperactivate the luciferase expression induced by 1 μM RA.

(D–F) Zfp423 is required for transcriptional regulation of endogenous RA target genes in response to RA. mRNA expression analysis of the RA target genes *Rar*β (D), *Crabp2* (E), and *Wnt6* (F) in F9 cells expressing shRNAs targeting *GFP*, *Zfp423*, or *Rxr*α after 1 μM RA stimulation for 48 hr is shown. Error bars represent SD of triplicate independent experiments.

(Figure S1). These results suggest that ZNF423 functions as a cofactor for RARα/RXRα transactivation.

Using a similar approach, we found that ZNF423 is required for activation of the RARE-Luc reporter induced by RAR-selective agonists for all isoforms: RARα, β, and γ (Figure S2). Consistent with these results, coexpression of ZNF423 sensitized the RA response in *Rar*α,β,γ triple-knockout (TKO) mouse embryonic fibroblasts (MEFs) reconstituted with each of the three RAR isoforms (Figure S3) (Epping et al., 2007). Furthermore, ZNF423 was also required for both reporter gene activation and growth inhibition induced by an RXR-selective ligand in mouse F9 and human SH-SY5Y neuroblastoma cells (Figures S4 and S5). Together, these results indicate that ZNF423 is a critical cofactor for all three retinoic acid receptors.

To further substantiate that *Zfp423* knockdown inhibits RA-induced transcription, we analyzed mRNA expression levels of a panel of eight bona fide RA target genes, including *Rar*β and *Crabp2*, in F9 cells. As expected, all eight target genes were drastically induced in control cells following exposure to RA (Figures 3D and 3E; Figure S6). However, induction of these genes was significantly inhibited in cells expressing sh*Zfp423*
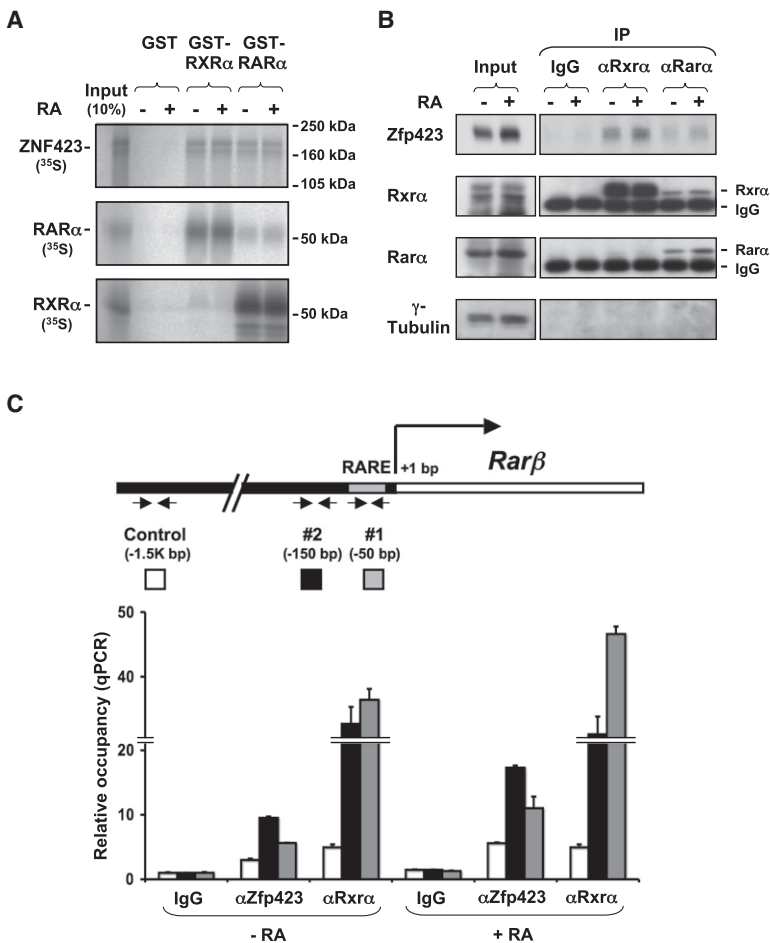
or sh*Rxr*α (Figures 3D and 3E; Figure S6). In addition, it has been shown that *Wnt6* expression is downregulated in F9 cells upon RA treatment (Eifert et al., 2006). We also observed this repression of *Wnt6* in control F9 cells in response to RA, but not in cells stably expressing sh*Zfp423* or sh*Rxr*α (Figure 3F). These results further demonstrate that ZNF423 is required for RXRα/RARα transcriptional regulation in response to RA.

In contrast, Zfp423 appeared not to be required for Rarα-mediated transcriptional repression in the absence of RA in F9 cells (Figure S6). Consistent with this, ZNF423 was not required for transcriptional repression of GAL4-luciferase by GAL4-RARα in the absence of RA (Figure S7).

Interaction of ZNF423 with Retinoic Acid Receptors In Vitro and In Vivo

To investigate the possible physical interaction between ZNF423 and RARα/RXRα, we performed GST pull-down experiments using recombinant GST fusions of RXRα, RARα, and in vitro-translated ZNF423. ZNF423 associated with GST-RXRα and GST-RARα in both the absence and presence of RA (Figure 4A). As a control for proper folding of the fusion proteins, heterodimerization between GST-RXRα and RARα or vice versa was observed, but homodimerization of RARα or RXRα was inefficient under the conditions used (Figure 4A). To determine whether ZNF423 binds RARα/RXRα in vivo, we performed coimmunoprecipitation experiments in parental F9 cells. Endogenous

A

B

C

Figure 4. ZNF423 Physically Associates with RARα/RXRα and the RARE Region of the *Rar*β Promoter

(A) In vitro binding of ZNF423 to RXRα and RARα. GST pull-down assay showing the interactions of in vitro-translated ^{35}S-labeled ZNF423 with recombinant GST-RXRα and GST-RARα independent of 1 μM RA is shown. The same protein preparations were used to demonstrate heterodimerization between GST-RXRα and in vitro-translated RARα and between GST-RARα and in vitro-translated RXRα.

(B) In vivo interaction of Zfp423 and the Rarα/Rxrα complex in mouse F9 cells. Rxrα and Rarα were immunoprecipitated (IP) using their specific antibodies (labeled as αRxrα and αRarα; IgG was used as a control) in the absence or presence of 1 μM RA. The precipitates were immunoblotted for Zfp423, Rxrα, Rarα, and γ-tubulin.

(C) Zfp423 is associated with the RARE region of the *Rar*β promoter. Chromatin immunoprecipitation analysis demonstrates the promoter occupancy by endogenous Zfp423 on the *Rar*β promoter in parental F9 cells. Quantitative PCR was used to show promoter occupancy using two primer sets in the proximity of the RARE and one control primer set located 1.5 kb distal to the RARE region. Specific enrichment of *Rar*β promoter occupancy was observed in the Zfp423 and Rxrα immunoprecipitates as compared to IgG control immunoprecipitate in both the absence and the presence of 1 μM RA. Error bars represent SD of triplicate measurements.

Rxrα and Rarα coimmunoprecipitated with endogenous Zfp423 (Figure 4B), indicating that Zfp423 and Rarα/Rxrα form a stable complex under physiological conditions in vivo. Consistent with the in vitro binding results, the association of Zfp423 with Rarα/Rxrα in F9 cells did not require RA but was slightly enhanced when cells were treated with 1 μM RA. This enhanced interaction in response to RA coincided with the slight increase in Zfp423 protein and mRNA levels after RA exposure (Figure 4B and data not shown).

Since ZNF423 directly associates with RXRα and is required for transactivation of all three RARs, we predicted that ZNF423 would also interact with RARβ and RARγ in addition to RARα. Indeed, all RAR isoforms were able to coimmunoprecipitate with ZNF423 in cells transfected with pSG5-RXRα/RARα either with or without pCS2-FLAG-ZNF423ΔN (Figure S8).

Zfp423 Interacts with the RARE Region of the *Rar*β Promoter

Since Zfp423 forms a stable complex with Rarα/Rxrα and is required for their transcriptional regulation in response to RA, we examined whether Zfp423 interacts with the promoters of bona fide RA target genes in chromatin. Using antibodies for Zfp423, Rxrα, or control IgG and primers sets flanking the RARE region of the *Rar*β promoter, we performed chromatin immunoprecipitation (ChIP) assays on F9 cells followed by quan-

titative PCR (qPCR). Rxrα, used as a positive control, showed strong and specific interaction with the RARE region (Figure 4C). Zfp423 was also significantly associated with the *Rar*β promoter in direct proximity to the RARE in both the presence and the absence of RA, while its occupancy in the region distal to the RARE was minimal (Figure 4C). The small increase in Zfp423 occupancy in the RARE region after RA treatment is consistent with the increased association between Zfp423 and Rarα/Rxrα and may be a consequence of the upregulation of Zfp423 levels as described above (Figure 4B).

ZNF423 Is Critical for Cell Growth and Differentiation of Neuroblastoma

RA is a neural differentiation agent, and RA signaling has been implicated in differentiation of human neuroblastoma cells (Reynolds et al., 1994; Sidell et al., 1983). Clinical trials have shown that RA treatment after completion of intensive chemoradiotherapy significantly improves survival in high-risk neuroblastoma patients (Matthay et al., 1999; Reynolds et al., 2003). Our finding that ZNF423 is a critical cofactor for RXRα/RARα and the notion that Zfp423 is involved in neural development in mice (Cheng and Reed, 2007; Cheng et al., 2007; Warming et al., 2006) suggested a possible role for ZNF423 in human neuroblastoma. Furthermore, ZNF423 is expressed in many neuroblastoma cell lines (Figures S11 and S12). Therefore, we investigated whether ZNF423 is causally involved in cell growth and differentiation of human neuroblastoma cell lines. In response to RA treatment, many neuroblastoma cell lines exhibited arrest of proliferation and/or morphological differentiation (Figure 5; Figure S9; data not shown). Similar to the RA-induced upregulation of *Zfp423* in F9 cells, we also observed a small increase of *ZNF423*
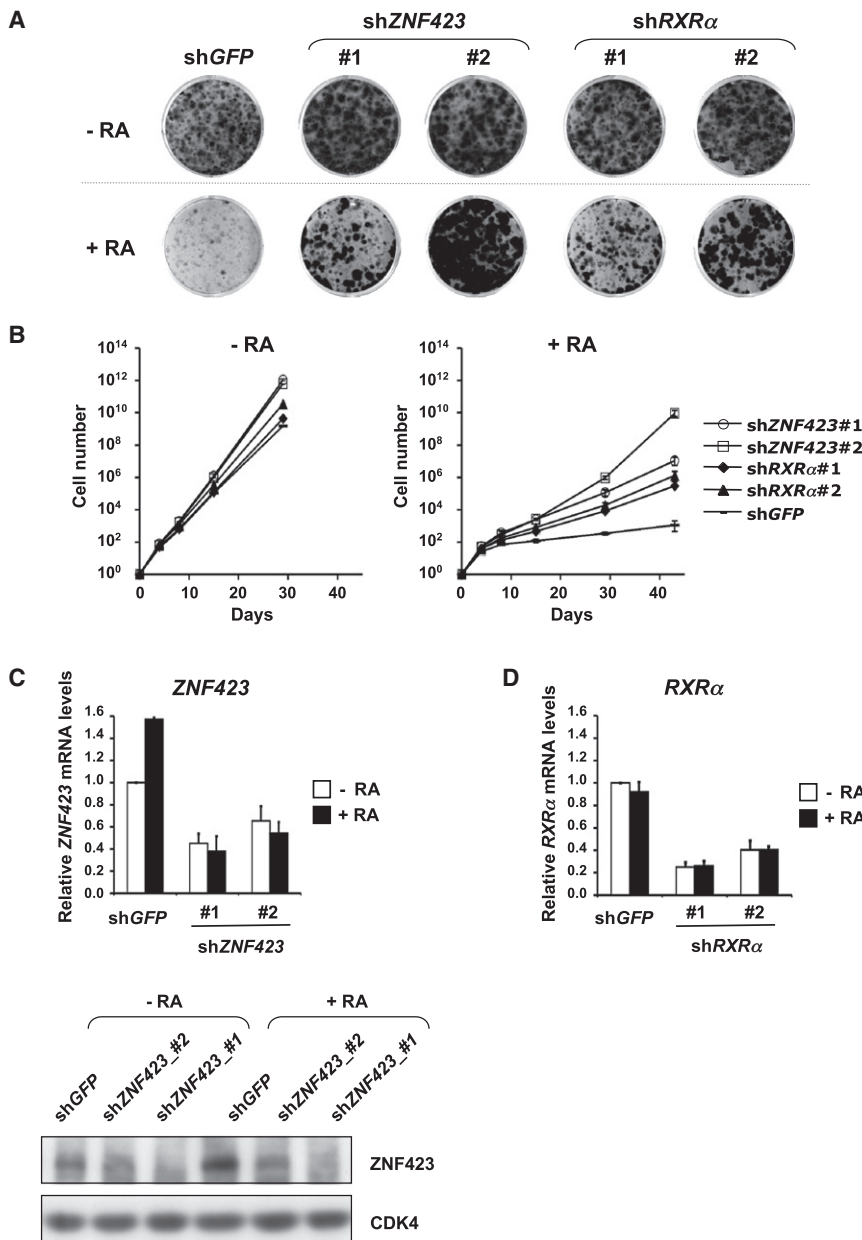
Figure 5. Suppression of _ZNF423_ Leads to a Growth Advantage and Resistance to RA-Induced Differentiation in Neuroblastoma

(A) Downregulation of _ZNF423_ or _RXRα_ in SH-SY5Y cells by RNAi results in a growth advantage in the absence of exogenous RA and resistance to RA-induced growth inhibition and differentiation. SH-SY5Y cells expressing shRNAs against control _GFP_, _ZNF423_, or _RXRα_ were grown in the absence or presence of 0.1 μM exogenous RA, after which cells were fixed, stained, and photographed. Cells were harvested after 20 days (untreated) or 35 days (RA treatment).

(B) Proliferation curves according to the 3T3 protocol of SH-SY5Y cells expressing shRNAs targeting _ZNF423_, _RXRα_, or _GFP_ in the absence (left) or presence (right) of 0.1 μM exogenous RA. Error bars represent SD of triplicate measurements.

(C) _ZNF423_ mRNA and protein levels were slightly induced in SH-SY5Y cells in response to 2 weeks of 0.1 μM RA treatment, and suppression of _ZNF423_ by shRNAs blocked this induction as well as the basal expression in the absence of RA. Error bars represent SD of triplicate measurements.

(D) Downregulation of _RXRα_ mRNA levels by shRNAs targeting _RXRα_ in SH-SY5Y cells (±0.1 μM RA). Error bars represent SD of triplicate measurements.

of several critical neural differentiation markers, the neurotrophic receptors _TRKA_ and _TRKB_ as well as the receptor for glial cell-derived neurotrophic factor (GDNF), _RET_. It has been shown that expression of these receptor kinases is induced upon RA treatment and that these receptors are causally involved in RA-induced differentiation in neuroblastoma cells in culture (Esposito et al., 2008; Kaplan et al., 1993; Peterson and Bogenmann, 2004). When control SH-SY5Y cells were exposed to RA for 2 weeks, mRNA levels of _TRKA_, _TRKB_, and _RET_ were induced (Figure 6). In contrast, the induction of these neurotro-

mRNA and protein in human SH-SY5Y neuroblastoma cells after RA treatment (Figure 5C). Downregulation of _ZNF423_ expression by multiple nonoverlapping shRNA vectors conferred resistance to the potent effects induced by RA in SH-SY5Y, SK-N-SH, and SK-N-FI neuroblastoma cells, which are non-_MYCN_-amplified (Figure 5 and data not shown). Similar results were also obtained in _MYCN_-amplified cells such as SK-N-BE, IMR32, and SK-N-AS cells (Figure S9 and data not shown), indicating that the role of _ZNF423_ in RA signaling in neuroblastoma cells is independent of _MYCN_ status. Consistent with the RA resistance caused by _ZNF423_ knockdown, the induction of the RA target genes _RARβ_ and _CRABP2_ was significantly inhibited in SH-SY5Y cells stably expressing shRNAs against _ZNF423_ or _RXRα_ (Figure 6).

To address whether _ZNF423_ knockdown interferes with neuroblastoma differentiation, we also examined the expression

phic receptors in cells expressing shRNAs against _ZNF423_ or _RXRα_ was significantly inhibited (Figure 6).

Conversely, enforced expression of _ZNF423_ in SH-SY5Y and SK-N-SH cells led to hypersensitivity to RA, as evidenced by growth inhibition (Figures S10A and S10B and data not shown). As expected, SH-SY5Y cells expressing exogenous _ZNF423_ induced higher levels of RA target genes and neural differentiation markers in response to RA treatment (Figure S10C). Moreover, expression of _ZNF423_ in RA-insensitive SHEP cells that have undetectable levels of _ZNF423_ restored the defective RA response (Figure 7; Figure S11). Collectively, these results demonstrate that _ZNF423_ is required for RA-induced differentiation in neuroblastoma cells.

Surprisingly, we also noticed a growth advantage in neuroblastoma cells expressing shRNAs against _ZNF423_ or _RXRα_ in the
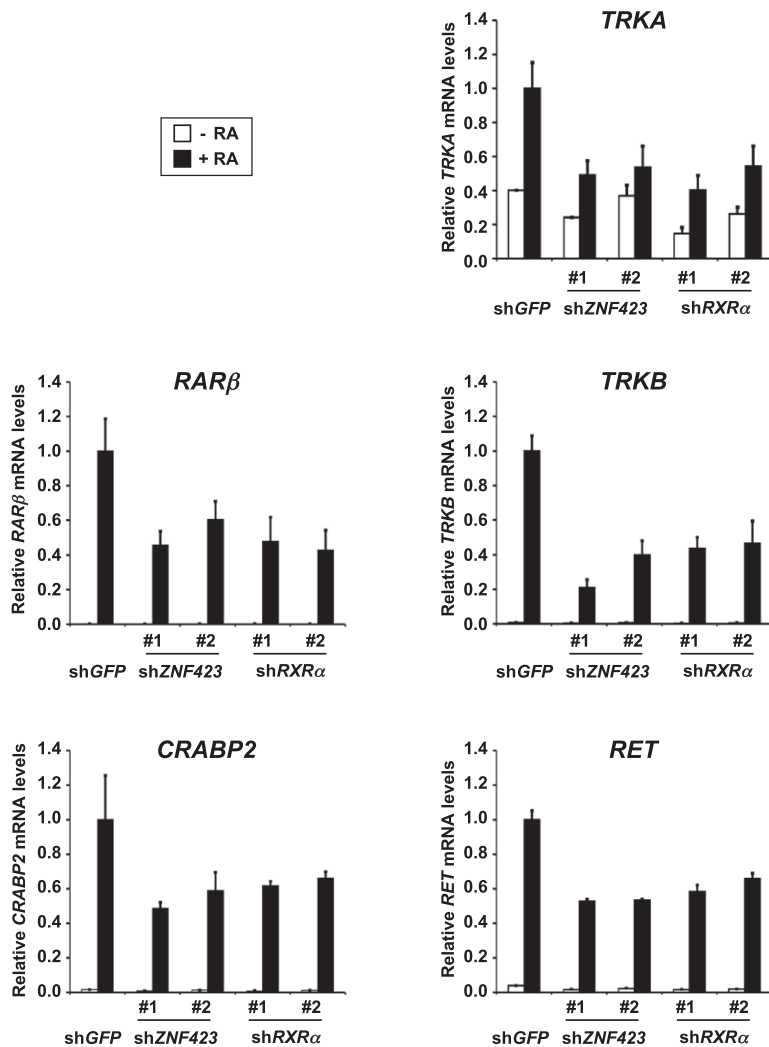
Figure 6. ZNF423 Is Required for Transcriptional Responses of RA Target Genes and Neural Differentiation Markers in Neuroblastoma

Suppression of *ZNF423* and *RXRα* in SH-SY5Y cells by RNAi leads to reduced expression of RA target genes and neural differentiation markers in both the absence and the presence of exogenous RA (see also Figure S4). mRNA levels of *RARβ*, *CRABP2*, *TRKA*, *TRKB*, and *RET* in SH-SY5Y cells expressing shRNAs targeting *GFP*, *ZNF423*, or *RXRα* were analyzed by qRT-PCR after the cells had grown in the absence or presence of 0.1 μM exogenous RA for 14 days. Error bars represent SD of triplicate measurements.

absence of exogenous RA (under normal growth conditions containing 8% FCS) (Figure 5 and data not shown). This growth advantage was also observed, but to a lesser extent, in IMR32 and SK-N-AS cells with *MYCN* amplification (data not shown). Conversely, ectopic expression of *ZNF423* in SHEP, SH-SY5Y, or SK-N-SH cells resulted in growth inhibition even when cultured in the absence of exogenous RA (Figure 7; Figure S10; data not shown). These effects of *ZNF423* knockdown and overexpression could be partially due to low concentrations of RA present in the serum-containing culture medium (see also Discussion).

Expression of *ZNF423* Is Prognostic for Survival of Neuroblastoma Patients

Given the role of ZNF423 in neuroblastoma growth and differentiation, we investigated a possible correlation between *ZNF423* expression and progression-free survival in human neuroblastoma patients. The initial analysis was performed using a cohort of 88 human neuroblastoma patients (72 without *MYCN* amplification and 16 with *MYCN* amplification; see also Tables S2 and S3) from the Academic Medical Center (AMC) in Amsterdam. The expression data were obtained by microarray analysis (Affymetrix platform) of the primary tumor samples of these

patients at the time of diagnosis, prior to any treatment. Using this cohort as a "training set," a cutoff value of *ZNF423* expression was determined using leave-one-out cross-validation (see also Experimental Procedures and Table S3). The patients were then classified into two groups based on *ZNF423* expression in their primary tumors using this cutoff value, and Kaplan-Meier analysis for progression-free survival was performed. Interestingly, a high level of *ZNF423* expression was associated with good outcome of patients (all stages combined), and low *ZNF423* expression was associated with poor outcome (Figure 8A, left; p = 2.9e-03). This potential prognostic value of *ZNF423* expression was also significant in the subset of 72 patients with tumors lacking *MYCN* amplification (Figure 8A, right; p = 4.5e-03) but appeared not to be statistically significant in the 16 patients with tumors having *MYCN* amplification (p = 0.494; data not shown), likely due to the small number of patients in this subgroup.

Next, we validated the prognostic value of *ZNF423* using a second independent cohort of 102 patients with metastatic neuroblastoma tumors lacking *MYCN* amplification (Asgharzadeh et al., 2006). Since the gene expression data of the second cohort were also based on the same microarray platform, the *ZNF423* expression values from both data sets were normalized, and the same *ZNF423* cutoff value determined in the AMC cohort was used for the validation study (see Supplemental Experimental Procedures). Similar results were obtained in the second cohort: a high level of *ZNF423* expression was associated with good outcome of patients in progression-free survival, and low *ZNF423* expression was associated with poor outcome (Figure 8C; p = 3.3e-04). Thus, these results validate the prognostic value of *ZNF423* in neuroblastoma. Furthermore, using this validated *ZNF423* cutoff value, multivariate analysis of the AMC cohort with other clinically relevant parameters showed that expression of *ZNF423* predicted survival independently of *MYCN* amplification and patient age (Figure 8B).
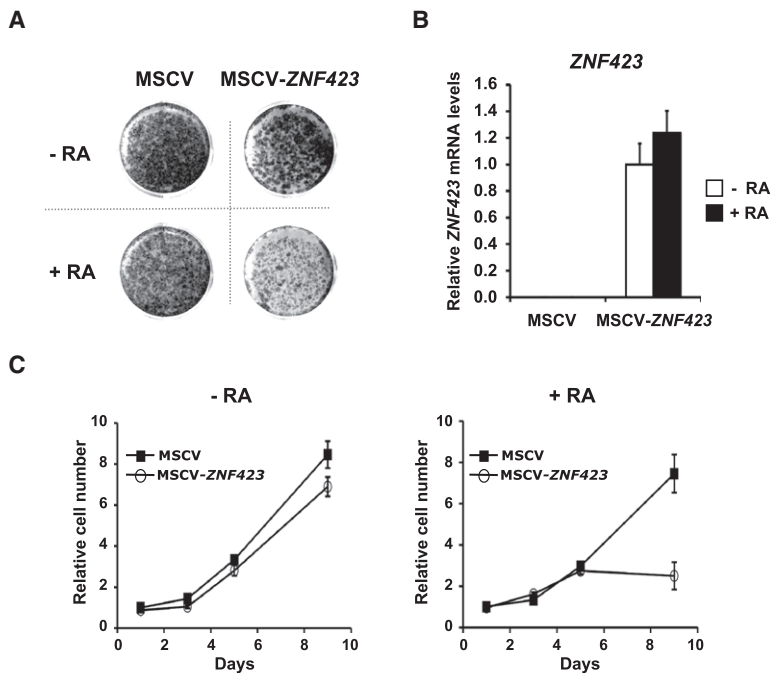
This prognostic value of *ZNF423* is further supported by a third independent data set of 101 neuroblastoma patients (81 without *MYCN* amplification and 20 with *MYCN* amplification) (Wang et al., 2006), where *ZNF423* expression was high in tumors of early-stage disease but lower in more advanced-stage disease

A

MSCV   MSCV-*ZNF423*

- RA

+ RA

B

C

Figure 7. Enforced Re-expression of *ZNF423* Restores RA Sensitivity in Neuroblastoma

(A) Re-expression of *ZNF423* in SHEP cells that have undetectable levels of *ZNF423* (see also Figure S11) leads to growth inhibition in the absence of exogenous RA and enhanced responses to RA. Cells retrovirally infected with MSCV control or MSCV-*ZNF423* were grown in the absence or the presence of 0.1 μM exogenous RA for 9 days, after which cells were fixed, stained, and photographed.

(B) Relative *ZNF423* mRNA levels in SHEP cells retrovirally infected with MSCV control or MSCV-*ZNF423* (±0.1 μM RA). Error bars represent SD of triplicate measurements.

(C) Graphical view of SHEP cell growth (±0.1 μM RA) based on crystal violet staining. Compared to control cells, cells infected with MSCV-*ZNF423* showed growth suppression and enhanced RA sensitivity. Error bars represent SD of triplicate measurements.

(Figure 8D, left; p = 3.1e-0.5). Similar correlation was also observed in the subset of 81 patients with tumors lacking *MYCN* amplification (Figure 8D, right; p = 3.1e-03). Since *MYCN*-amplified tumors existed only in stage 4 of this cohort, such correlation analysis of *ZNF423* expression and stages could not be performed in this subgroup.

When we expressed ZNF423 in human neuroblastoma cells and performed global gene expression analysis, we found that 852 genes were >2-fold regulated by ZNF423 (NCBI GEO accession number GSE14627). Of these 852 genes, 142 (see Table S4) were also significantly differentially expressed in the "*ZNF423* high" versus the "*ZNF423* low" cohort of 102 neuroblastomas (Asgharzadeh et al., 2006). Randomization analyses demonstrate that the genes that respond to *ZNF423* expression in vitro are also statistically significantly associated with *ZNF423* expression in patient tumors (Figure S13).

In summary, these multiple expression analyses in patient tumors indicate that expression of *ZNF423* is prognostic for outcome in human neuroblastoma patients.

DISCUSSION

We identified ZNF423 in an unbiased genome-wide RNAi screen for additional components of the RA signaling pathway and found that it is a critical cofactor of RA-induced transcription that interacts with RARα/RXRα. Subsequently, we demonstrated that ZNF423 is critical for growth and differentiation of neuroblastoma cells and identified the prognostic value of *ZNF423* expression in long-term survival of human neuroblastoma patients.

Previous studies have indicated that ZNF423 is a multifunctional transcriptional regulator that uses distinct sets of its zinc fingers to regulate different signaling pathways. In this study, we provide several lines of evidence for a function of ZNF423 in positively regulating cellular responses to RA. First, suppression of *ZNF423* expression by RNAi conferred resistance to RA-induced differentiation in mouse teratocarcinoma cells, mouse ES cells, and a panel of human neuroblastoma cells. Conversely, ectopic expression of ZNF423 in RA-insensitive neuroblastoma cells with undetectable levels of *ZNF423* restored the RA response. Second, suppression of *ZNF423* by RNAi in mouse teratocarcinoma and human neuroblastoma cells impaired the full induction of endogenous RA target genes in response to RA treatment, indicating that ZNF423 is a critical cofactor required for RARα/RXRα transactivation. Third, ZNF423 interacts with RARα/RXRα both in vitro and in vivo, and ZNF423 associates with RAREs of the promoter region of RA target genes in ChIP experiments.

The interaction between ZNF423 and RARα/RXRα does not require RA either in vitro or in vivo, and consistent with this, we found that Zfp423 constitutively associated with the RARE of the *Rar*β promoter. However, ZNF423 does not appear to be required for repression by the unliganded RAR (Figure S7). Moreover, in line with previous observations (Hata et al., 2000), we did not detect any intrinsic ability of ZNF423 to activate transcription (data not shown). Taking these findings together, we propose a role for ZNF423 as a transcriptional intermediary factor for RARα/RXRα: it constitutively associates with the promoters of RA target genes and may prime the RA receptors to recruit other essential factors required for RARα/RXRα transactivation in response to RA. Identification of these critical factors recruited by ZNF423 will yield further insights into the regulation of RARα/RXRα transactivation.

Previous studies have indicated that Zfp423 plays an important role in neural development in mice: deletion of *Zfp423* results in severe cerebellar defects and impaired olfactory neurogenesis as well as CNS midline patterning in mice (Cheng and Reed, 2007; Cheng et al., 2007; Warming et al., 2006). These effects of Zfp423 appear to coincide with a central role of RA signaling in neuronal differentiation and CNS patterning in animals (Maden, 2002; McCaffery et al., 2003). *Rar* knockout mice exhibit neural crest defects and disorganized hindbrain, and these effects can be mimicked by the use of a pan-RAR antagonist. These in vivo phenotypes are consistent with the
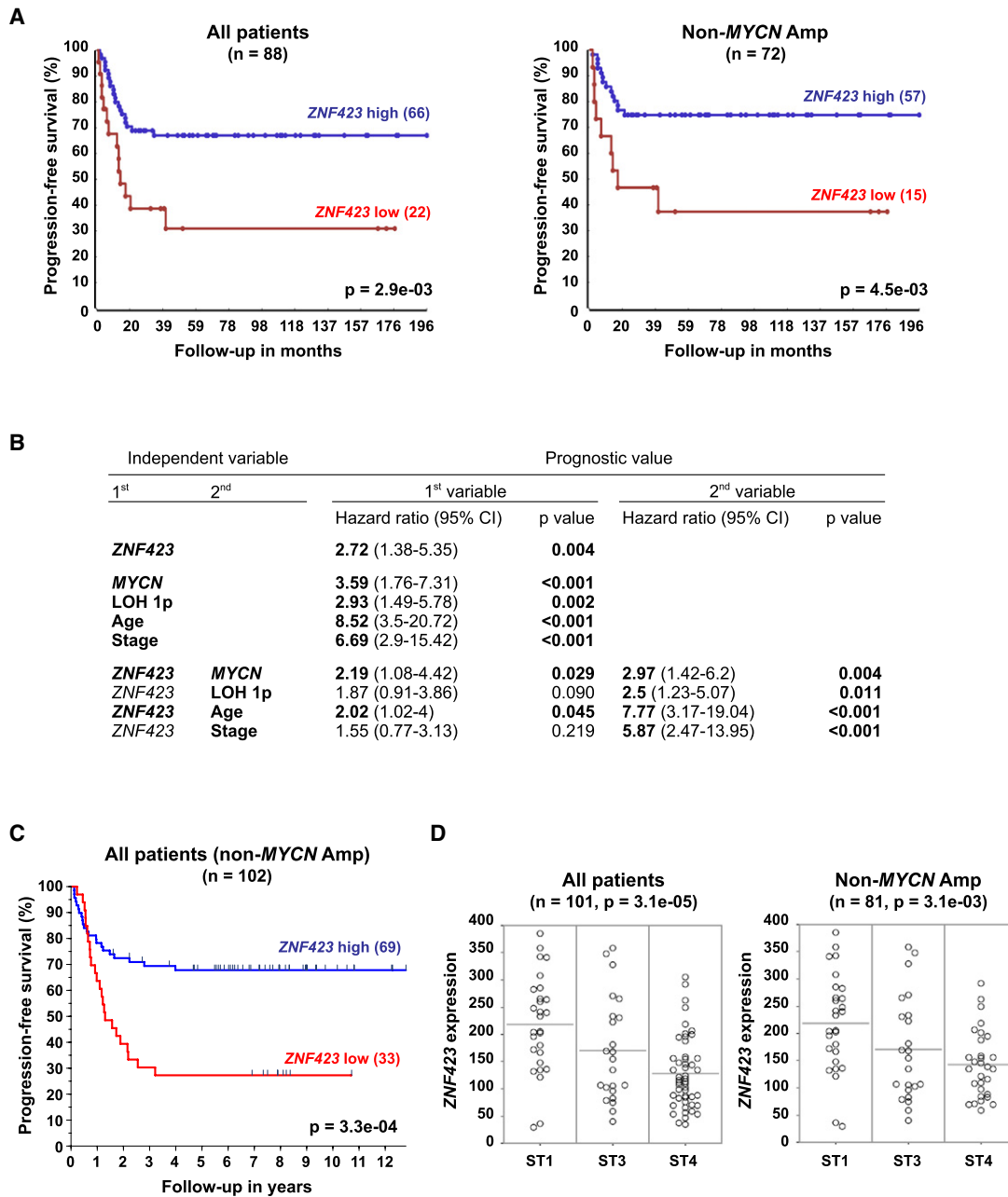
Figure 8. Expression of *ZNF423* Is Prognostic for Survival of Human Neuroblastoma Patients

(A) Kaplan-Meier analysis of the Academic Medical Center (AMC) cohort documenting increased progression-free survival of neuroblastoma patients with tumors that have high *ZNF423* expression (*ZNF423* high) versus patients with tumors that have low *ZNF423* expression (*ZNF423* low), using the *ZNF423* cutoff value determined as described in the text. The analysis in the left diagram was performed in 88 tumors including all stages (stage 1 to 4S); the right diagram presents a subanalysis of 72 tumors lacking *MYCN* amplification.

(B) Multivariate analysis of the AMC cohort using *ZNF423* expression and other clinically relevant parameters including *MYCN* amplification, loss of heterozygosity of chromosome 1p (LOH 1p), age, and stage (see also Experimental Procedures). Expression of *ZNF423* predicted survival independently of *MYCN* amplification and patient age. CI denotes confidence intervals; statistically significant parameters are shown in bold. All patients (n = 88) were included in the entire analysis except for the studies with LOH 1p (n = 82) due to the undetermined LOH 1p status in 6 patients.

(C) Kaplan-Meier analysis of progression-free survival for a second independent set of 102 patients diagnosed with metastatic neuroblastomas lacking *MYCN* amplification (Asgharzadeh et al., 2006). These patients were classified using the same *ZNF423* cutoff value determined from the AMC cohort.

(D) Decreased expression of *ZNF423* correlates with advanced tumor stages in a third independent set of 101 neuroblastoma patients (Wang et al., 2006). The analysis in the left diagram was performed in all of the patients (81 without *MYCN* amplification and 20 with *MYCN* amplification); the right diagram presents a subanalysis of the 81 tumors lacking *MYCN* amplification. ST1, stage 1 (n = 28*); ST3, stage 3 (n = 23); ST4, stage 4 (n = 53). *One stage 2 patient was included in the stage 1 group due to the low patient number in the stage 2 group (n = 1).

effects of ZNF423 on RA signaling in neuronal cell types reported here.

In the absence of exogenous RA, we also observed effects on cell proliferation and differentiation by *ZNF423* knockdown and overexpression in many of the neuroblastoma cell lines. It is possible that the endogenous amount of RA in the regular 8% FCS growth medium is sufficient to activate RA signaling at a low level in these human neuroblastoma cells. Alternatively, other pathways such as the BMP and OLF/EBF pathways, in which ZNF423 is also causally involved, could be important for neuroblastoma genesis. BMP2 treatment results in growth arrest and differentiation in human neuroblastoma cell lines, and OLF/EBF transcription factors have also been implicated in neuroblastoma differentiation (Nakamura et al., 2003; Persson et al., 2004). In fact, a synergistic effect of RA and BMP6 on differentiation of human neuroblastoma cells has been demonstrated in culture (Sumantran et al., 2003). As the common mediator for both the RA and BMP pathways, ZNF423 might be the critical factor contributing to this synergy. We therefore cannot rule out the possibility that other RA-independent pathways are also responsible for the prognostic value of *ZNF423* expression.

Since RA induces growth arrest, differentiation, and apoptosis, one might speculate that ZNF423, being an essential mediator of the RA response, has tumor suppressor-like properties in neuroblastoma. However, we did not observe loss of heterozygosity at chromosome 16q12, the *ZNF423* locus, in a panel of 88 human neuroblastomas, nor did we find that human neuroblastoma cell lines that do not express *ZNF423* could be made to re-express the gene after treatment with DNA demethylating agents (data not shown).

RA-based therapies are also used in leukemias, most notably in acute promyelocytic leukemia (APL). However, we did not observe any *ZNF423* expression in two leukemia cell lines, including one APL (Figure S11). This is in agreement with the neural-restricted expression pattern of *ZNF423* (http://symatlas.gnf.org/SymAtlas/) and the neural-specific phenotype of *Zpf423* knockout mice (Cheng and Reed, 2007; Cheng et al., 2007; Warming et al., 2006). Thus, ZNF423 may not be a regulator of the RA response in leukemia. However, since the ZNF gene family consists of some 700 members, it is possible that another ZNF family member is required for RA responses in other cell types.

Remarkably, in three independent gene expression data sets of over 300 neuroblastomas, we found that low expression of *ZNF423* predicted poor outcome. Multivariate analysis shows that *ZNF423* prognostic value is independent of *MYCN* status (Figure 8). These findings are significant since *MYCN* amplification only accounts for a subset of aggressive neuroblastoma tumors, while the remainder lack consistently identifiable biomarkers. *ZNF423* expression could therefore serve as a prognostic biomarker for neuroblastoma tumors independent of *MYCN* amplification.

In summary, our data indicate that low *ZNF423* expression is associated with poor outcome in neuroblastoma and clearly implicate ZNF423 in RA signaling. Expression levels of ZNF423 could significantly affect responses to both endogenous and pharmacological concentrations of RA in cancer patients, which may in turn influence the outcome of neuroblastoma. Therefore, *ZNF423* may also be a biomarker predicting responses to

RA-based therapies, which are increasingly being used to treat neuroblastoma. Additional clinical studies will be needed to validate this potential predictive power of *ZNF423* expression for retinoid-based therapies in human neuroblastoma.

EXPERIMENTAL PROCEDURES

shRNA Library
The NKI mouse shRNA library (containing 28,256 shRNA vectors that target 14,128 mouse genes) was constructed into the pRISC retroviral vector, which is derived from pRETRO-SUPER with an additional chloramphenicol resistance marker under regulation of the *TET* promoter (Brummelkamp et al., 2002). The details of this library can be found in the Supplement Data and can also be viewed at http://www.screeninc.nl/.

Transfections and Reporter Assays
Transfections were carried out using calcium phosphate precipitation. RARE-Luc reporter assays were performed in DMEM supplemented with charcoal-stripped FCS (HyClone) essentially as described previously (Epping et al., 2007).

GST Pull-down Assays, Western Blotting, Coimmunoprecipitation, and Chromatin Immunoprecipitation
These experiments were performed according protocols described previously (Epping et al., 2005). Normal rabbit IgG, anti-RXRα (D-20), and anti-ZNF423 (H-105) were used for ChIP. The sequences of the primer sets used for ChIP-qPCR analysis are listed in Table S1.

Patient Samples
For all three independent studies presented, the expression data were obtained using Affymetrix microarray analyses on untreated primary tumor samples at the time of diagnosis. For the cohort of 88 neuroblastoma patients from the AMC (Amsterdam), material was obtained during surgery and immediately frozen in liquid nitrogen. *MYCN* amplifications and loss of heterozygosity of chromosome 1p (LOH 1p) were determined using Southern blot analysis of tumor material and lymphocytes from the same patient. Patient samples for the other two cohorts were as described previously (Asgharzadeh et al., 2006; Wang et al., 2006). Written informed consent was obtained from patients' parents or guardians in accordance with Institutional Review Board policies and procedures for research dealing with tumor specimens and clinical information. The Institutional Review Board at Childrens Hospital Los Angeles and the Medical Ethics Committee of the AMC approved the study.

Statistical Analysis
To determine the optimal value to set as a cutoff for *ZNF423* expression in the AMC cohort, leave-one-out cross-validation was used. The neuroblastoma patients were sorted based on expression of *ZNF423* and subsequently divided into two groups based on the expression value of each patient. For each group separation (higher or lower than the current *ZNF423* expression), the log-rank significance was calculated. The best p value out of the sequence was then used to represent the final gene expression cutoff value for *ZNF423*. This cutoff value was validated using a second independent set of 102 patients (Asgharzadeh et al., 2006) (Supplemental Experimental Procedures).

Progression-free survival was measured for all outcome analyses presented in this study using the same *ZNF423* cutoff value. The definition of an event for the AMC cohort (all stages) was progressive disease, recurrent disease, or death of the patient. For the second cohort with metastatic neuroblastomas lacking *MYCN* amplification, disease progression was defined as development of any new lesion, >25% increase of any measurable tumor mass, or previously negative bone marrow becoming positive for tumor cells.

For multivariate analysis, Cox regression calculations on progression-free survival were performed using SPSS version 15.0. For these calculations, single covariates (*ZNF423*, *MYCN*, LOH 1p, stage, age) as well as double covariates in a nonsequential model (*MYCN*, LOH 1p, stage, age) in combination with the *ZNF423* cutoff were used. The variable "stage" consisted of two groups based on the International Neuroblastoma Staging System: (1) ST1, 2, 3, and 4S and (2) ST4. The variable "age" (of diagnosis) consisted of two

groups: (1) ≤18 months and (2) >18 months. Hazard ratios, p values, and 95% confidence intervals were part of the SPSS output.

ACCESSION NUMBERS

The gene expression data for SH-SY5Y cells with and without overexpression of *ZNF423* are available at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE14627.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, thirteen figures, and four tables and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(09)00075-0.

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