ORIGINAL RESEARCH ARTICLE

Distinct mechanisms of regulation of the ITGA6 and ITGB4 genes by RUNX1 in myeloid cells†

Running Title: Regulation of integrin genes by RUNX1

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ABSTRACT

Integrins are transmembrane adhesion receptors that play an important role in hematopoiesis by facilitating interactions between hematopoietic cells and extracellular matrix components of the bone marrow and hematopoietic tissues. These interactions are important in regulating the function, proliferation and differentiation of hematopoietic cells, as well as their homing and mobilization in the bone marrow. Not surprisingly altered expression and function of integrins plays a key role in the development and progression of cancer including leukemias. However, the regulation of integrin gene expression is not well characterized and the mechanisms by which integrin genes are disrupted in cancer remain unclear. Here we demonstrate for the first time that a key regulator of hematopoiesis, RUNX1, binds to and regulates the promoters of both the ITGA6 and ITGB4 genes in myeloid cells. The ITGA6 and ITGB4 integrin genes form the α6β4 integrin receptor. However our data indicates that RUNX1 functions differently at these two promoters. RUNX1 regulates ITGA6 through a consensus RUNX1 binding motif in its promoter. In contrast, although the ITGB4 promoter is also activated by RUNX1, it does so in the absence of a recognized consensus RUNX1 binding motif. Further, our data suggest that regulation of ITGB4 may involve interactions between the promoter and upstream regulatory elements.

Key words: Integrin; RUNX1; Gene expression; Transcriptional regulation

Introduction

RUNX1 is a member of the RUNX family of transcription factors, which regulate cellular differentiation and commitment. RUNX1 is expressed in a range of cell types, but most notably, plays a pivotal role in hematopoietic development as well as in the function of mature hematopoietic cells. Not surprisingly then, disruption of the RUNX1 gene contributes to the development of leukemia, with point mutations (Osato et al., 1999), gene amplification (Dal Cin et al., 2001; Harewood et al., 2003; Mikhail et al., 2002; Niini et al., 2000) and chromosomal translocations (De Braekeleer et al., 2011) frequently observed in leukemia. In fact the *RUNX1* gene (previously known as *Acute Myeloid Leukemia 1* or *AML1*) was first identified following characterization of the t(8;21) chromosomal translocation found in 10-15% of acute myeloid leukemia (AML) (Miyoshi et al., 1991). This translocation fuses the N-terminal region of the *RUNX1* gene on chromosome 21q to the *RUNX1T1* gene *(also known as Eight-Twenty One* or *ETO*) on chromosome 8q, generating a RUNX1-ETO chimeric protein (reviewed in (Lam and Zhang, 2012; Licht, 2001)). The fusion protein contains the first 177 amino acids of the RUNX1 transcription factor encompassing the DNA binding domain, and the last 575 amino acids of the 604 amino acid ETO protein (Miyoshi et al., 1993). It is clear from a number of studies that disruption of RUNX1 function results in abnormal hematopoiesis (Speck and Gilliland, 2002). *Runx1* knockout (*Runx1*$^{-/-}$) mice are deficient in fetal liver hematopoiesis and die during embryogenesis (Okuda et al., 1996). *Runx1-Runx1t1* knock-in mice, which express RUNX1-ETO, have a similar phenotype, displaying a deficiency in fetal hematopoiesis (Yergeau et al., 1997). Furthermore, mice transplanted with RUNX1-ETO expressing hematopoietic stem cells display abnormal myeloid lineage development (de Guzman et al., 2002). Ectopic expression of the RUNX1-

ETO protein also inhibits differentiation of myeloid progenitor cells in culture with overexpression of RUNX1 relieving this repression (Kitabayashi et al., 1998).

The RUNX1 transcription factor contains an N-terminal DNA binding region, called the Runt homology domain (RHD) and a C-terminal regulatory domain. RUNX1 binds to DNA as a heterodimer with the Core Binding Factor β (CBFβ) protein (Ogawa et al., 1993), which regulates RUNX1 DNA binding and function. Over the last twenty years, a considerable body of work has accumulated characterizing regulatory targets of RUNX1, with RUNX1 largely, but not exclusively, characterized as a transcriptional activator, functioning in co-operation with other transcription factors (Otto et al., 2003). Characterization of *bona fide* RUNX1 target genes has demonstrated that RUNX1 can regulate gene expression by binding to the well-defined consensus sequence TGT/cGGT, located within promoter or enhancer elements. Some of the best characterized of these target genes encode cytokines and cytokine receptors important for hematopoietic differentiation and function (Cockerill et al., 1996; Follows et al., 2003; Oakford et al., 2010). More recently, however, genome-wide approaches have enabled the identification of the extensive transcriptional programs under the control of RUNX1. A number of these studies have identified an important role for RUNX1 in regulating cell adhesion and migration programs (Lie et al., 2014; Michaud et al., 2008; Wotton et al., 2008). Further, RUNX1 regulation of several genes encoding cell adhesion molecules has been reported, including VLA-4 (also known as α4β1 (Ponnusamy et al., 2014)), CD44 (Peterson et al., 2007), ITGAL (Puig-Kroger et al., 2003) and PSGL1 (Ponnusamy et al., 2015).

The interaction of adhesion molecules on hematopoietic progenitor and stem cells with the bone marrow extracellular matrix (ECM) and stromal cells is important for the homing and

mobilization of these cells in the bone marrow and is also important for maintaining hematopoietic homeostasis (Prosper and Verfaillie, 2001). A range of molecules is important for these cell-cell and cell-ECM interactions, including the integrin receptors. The integrins are a large family of heterodimeric receptors that influence cell adhesion, proliferation, differentiation, migration, cell survival and signal transduction (reviewed in (Takada et al., 2007)). The integrin receptors α4β1 (Papayannopoulou and Nakamoto, 1993; Scott et al., 2003) and α5β1 (Levesque et al., 1995; Van der Loo et al., 1998; Wierenga et al., 2006) are also known as VLA-4 and VLA-5, respectively. Expression of α4 integrin has been correlated with mobilization and homing of hematopoietic stem cells in the bone marrow (Prosper et al., 1998), with deletion or inhibition of α4 integrin resulting in the mobilization and accumulation of hematopoietic stem cells in the peripheral blood and defective homing of the hematopoietic stem cells into the bone marrow (Papayannopoulou and Nakamoto, 1993; Scott et al., 2003). Similarly, β1 null hematopoietic stem cells fail to engraft in irradiated recipient mice due to impaired homing to the bone marrow (Potocnik et al., 2000). Further, altered integrin expression in leukemia has been suggested as a contributing factor to the development of the disease due to disruption of the interaction between hematopoietic cells and the bone marrow stroma (Brouwer et al., 2001; Csanaky et al., 1997; Delforge et al., 2004; Geijtenbeek et al., 1999; Verfaillie et al., 1992).

Altered expression of a number of integrins has been demonstrated in genome-wide studies analyzing cells or leukemic samples in which RUNX1 is disrupted (Tanaka et al., 2012; Valk et al., 2004), suggesting that members of this family may be regulated by RUNX1. For example, a microarray study examining the gene expression profiles of 285 individuals with AML, which clustered samples according to their molecular signatures, found that the ITGB4 gene is significantly upregulated in samples containing the t(8;21) chromosomal translocation

(Valk et al., 2004). Similarly, *ITGB4* was upregulated in association with the t(8;21) translocation in a microarray study examining gene expression profiles of leukemic cells containing the t(8:21) and inv(16) chromosomal rearrangements (Ichikawa et al., 2006). Here we show that RUNX1 binds to and activates the ITGB4 and ITGA6 promoters in myeloid cells. These genes encode the β4 and α6 integrins that heterodimerize to form the α6β4 receptor. We show that RUNX1 regulates the ITGA6 promoter through a consensus binding motif in its promoter and that RUNX1 overexpression increases endogenous ITGA6 expression. However, our data suggest that regulation of the ITGB4 gene is more complex with RUNX1 activating the promoter in the absence of a recognized RUNX1 binding motif, and functioning at the promoter through an alternative mechanism.

Materials and Methods

Plasmids

pCMV5-AML-1B (RUNX1) and pCMV5-AML1-ETO (RUNX1-ETO) plasmids were obtained from Addgene and have been previously described (Meyers et al., 1995). RcCMV (CMV) was acquired from Invitrogen.

The ITGB4 and ITGA6 promoter plasmids were created by cloning the DNA sequence upstream from the transcription start site of the integrin genes into a firefly luciferase reporter plasmid, pXPG (Bert et al., 2000). Briefly, primers were designed to: -1199 bp to +144 bp and -675 bp to +242 bp of the transcription start site of ITGB4 and ITGA6, respectively (accession numbers: NM_000213 and NM_001079818 aligned to human reference genome hg19) with *XhoI* and *HindIII* restriction enzyme sites incorporated into the primers for cloning into the pXPG plasmid (Table 1). The integrin promoters were PCR-amplified from genomic DNA isolated from K562 cells using GoTaq Green master mix (Promega) or

Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs), supplemented with 2% DMSO. The PCR products were purified, digested with *XhoI* and *HindIII* enzymes and ligated into the pXPG plasmid. Identity of the constructs was confirmed by sequencing.

The deletion constructs were cloned using primers detailed in Table 1, but using the respective pXPG parental plasmid as template DNA in the PCR reaction. PCR products were digested with specific restriction enzymes and ligated into the pXPG plasmid. The Enh-1 and Enh-2 regions were cloned into the -176/+144 ITGB4 plasmid at *BamHI* and *XhoI* sites (Table 1).

The pTracer-RUNX1 plasmid was generated by subcloning the RUNX1b cDNA from pSCOT-AML1b (Michaud et al., 2008) into pTracer-CMV/Bsd (Life Technologies).

Site-directed Mutagenesis

Potential RUNX1 binding sites in the ITGB4 and ITGA6 promoters were mutated to create restriction enzyme recognition sites using PCR. Briefly, two sets of primers were designed to amplify 5' and 3' regions relative to the RUNX1 binding sites, with the primers designed to incorporate either a *BglII* site or *EcoRV* site, which overlapped the RUNX1 binding sites in ITGB4 and ITGA6 promoters, respectively (Table 1). Integrin promoter regions were amplified from respective pXPG parental plasmid DNA using GoTaq Green master mix (Promega) or Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs), supplemented with 2% DMSO. PCR products were purified, digested with restriction enzymes and ligated into the pXPG plasmid. Plasmid identify and mutation of the potential binding site was confirmed by sequencing.

Cell Culture

K562, KG-1a and Kasumi-1 cells were obtained from American Type Culture Collection (ATCC) and cultured in RPMI as described previously (Oakford et al., 2010). K562 and KG-1a cells were subcultured every 2-3 days and were maintained between $1x10^5$ and $1x10^6$ cells/mL. Kasumi-1 cells were subcultured once a week and maintained between $2.0x10^5$ and $2.0x10^6$ cells/mL. All cell lines were cultured in a humidified incubator at 37˚C and 5% CO_2.

Transfection and luciferase reporter assay

K562 cells ($4.5x10^6$) were transfected with 5 μg of reporter plasmid with either 5 μg of CMV plasmid, RUNX1 or RUNX1-ETO expression plasmids at 270 V and 950 μF using a Bio-Rad Gene Pulser X Cell as previously described (Holloway et al., 2000). At 24 h post-transfection, cells were harvested, protein isolated, quantitated by Bradford assay (Bio-Rad, USA) and 50 μg of protein was analyzed for luciferase activity (Luciferase assay kit, Promega) using a Turner Biosystems Veritas Microplate Luminometer.

Generation of Stable Cell lines

pTracer-RUNX1 and pTracer-CMV/Bsd were linearized using ScaI and then purified by phenol chloroform extraction and ethanol precipitation. K562 cells were transfected with the linearized RUNX1 or control plasmid and transfection confirmed by analysis for GFP fluorescence using flow cytometry after 24 h. Cells were treated with 20 μg/ml of Blasticidin (Life Technologies, USA) every two days for 10 days to select for transfected cells. GFP positive cells were identified by flow cytometry and plated one cell per well in a 96-well plate in media containing 10 μg/ml of Blasticidin. Once confluent, GFP positive clones were selected and further cultured in 6 well plates and RNA isolated for gene expression analysis.

RNA isolation and quantitative PCR analysis

Total RNA was isolated using Tri-Reagent (Sigma) and reverse transcribed using Superscript III Reverse Transcriptase (Life Technologies, USA) and 50 ng cDNA was amplified by SYBR Green PCR on the Rotor-Gene 2000 real-time cycler (Corbett Research, Australia) using the QuantiTect SYBR Green PCR kit (Qiagen, USA), as previously described (Brettingham-Moore et al., 2005). Cycling conditions were as follows: 95˚C for 15 minutes; 94˚C for 15 seconds and 60˚C for 1 minute for 40 cycles, acquiring on channel 1, followed by melt analysis from 60˚C to 95˚C. cDNA was amplified using primers described in Table 2. PCR was conducted in parallel using human GAPDH mRNA primers (Table 2) to normalize for discrepancies in cDNA synthesis and RNA input. To correlate the threshold (Ct) values from the amplification plots to copy number, a standard curve was generated for each primer pair with serial dilutions of the PCR product. PCR product melt curves were analyzed and the products were visualized by agarose gel electrophoresis to ensure a single PCR product was generated.

Nuclear extracts and western blotting

Nuclear extracts were prepared from K562, KG-1a and Kasumi-1 cells as described previously (Brettingham-Moore et al., 2005). Nuclear proteins were separated by SDS-PAGE through 12% polyacrylamide, transferred onto nitrocellulose membrane and subjected to western blot analysis using anti-RUNX1 (Santa Cruz Biotechnology, sc-8563) and anti-ETO (Santa Cruz Biotechnology, sc-9737) antibodies with the corresponding peroxidase-conjugated secondary antibodies. Proteins were visualized using the Supersignal West Pico Chemiluminescent kit (Pierce, USA) following the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed as previously described (Chen et al., 2005). Briefly cells ($7.5x10^6$ cells) were fixed with 1% formaldehyde, and chromatin was fragmented to a size range of 100-500 bp. Solubilized chromatin was immunoprecipitated with 5 μg of RUNX1 antibody (Santa Cruz Biotechnology, sc-8563) and 5 μg of ETO antibody (Santa Cruz Biotechnology, sc-9737). Immune complexes were recovered using salmon sperm DNA / protein A-agarose, washed and eluted. Following reversal of the cross-links and proteinase K treatment, the immunoprecipitated DNA was purified by phenol-chloroform extraction and ethanol precipitation. DNA was resuspended in 50 μL of MilliQ water and amplified by quantitative PCR using primers designed to the ITGB4 promoter, ITGB4 Enh-1 and Enh-2 regions, ITGA6 promoter and Rhodopsin promoter (Table 2).

Results

RUNX1 regulates the ITGB4 and ITGA6 promoters

Altered expression of the ITGB4 gene has previously been reported in microarray studies examining cells containing the t(8;21) translocation (Ichikawa et al., 2006; Valk et al., 2004). Therefore, expression of *ITGB4* was examined in Kasumi-1 myeloid cells which contain the t(8;21) translocation, the KG1a myeloid cell line, which is similarly a CD34+ AML-derived cell line and the K562 erythroleukemic cell line. All three cell lines express RUNX1 mRNA, as measured by quantitative PCR analysis, but only Kasumi-1 cells express RUNX1-ETO mRNA, produced by the t(8;21) translocation (Fig. 1A). ITGB4 mRNA levels were higher in Kasumi-1 cells compared to both KG1a and K562 cells (Fig. 1B). The β4 subunit encoded by *ITGB4* heterodimerizes exclusively with α6 (encoded by *ITGA6*) to form the α6β4 receptor. Therefore, levels of ITGA6 mRNA were also determined in these cell lines. *ITGA6* expression was comparable in Kasumi-1 and KG1a cells, but like *ITGB4*, expressed at very

low levels in the K562 cells (Fig. 1B). These data are in keeping with publicly available gene expression analysis in primary leukemic cells compared with healthy cells (Fig. 1C,D), which reveals increased expression of *ITGB4* but not *ITGA6* in the presence of the t(8;21) translocation (Hebestreit et al., 2012).

Since *ITGB4* levels are altered in cells containing the RUNX1-ETO protein, the ability of RUNX1 and RUNX1-ETO to regulate the ITGB4 promoter was examined using reporter assays. The ITGA6 promoter was also examined for comparison. Luciferase reporter constructs containing the promoters of the respective genes were generated and transfected into K562 cells. A region spanning from -1199 upstream to +144 bp downstream of the ITGB4 transcription start site was cloned into a pXPG luciferase reporter construct for analysis (Fig. 2A). This region contains 7 potential RUNX1 binding motifs, as determined by the MatInspector tool in the Genomatix bioinformatics suite V8.2 (http://www.genomatix.de/). In addition, the region from -675 bp to + 242 bp of the ITGA6 gene was cloned into pXPG. This region contains 9 potential RUNX1 binding motifs as determined using MatInspector (Fig. 2A). K562 cells were transfected with the pXPG-ITGB4 luciferase reporter construct (-1199/+144 ITGB4), either with or without a RUNX1 expression plasmid. Transfection of K562 cells with the RUNX1 expression plasmid resulted in increased expression of RUNX1 as detected by western blotting (Fig. 2B). Basal activity of the ITGB4 promoter reporter was detected in K562 cells and this activity increased approximately 28-fold following overexpression of RUNX1 (Fig. 2C). Transfection of K562 cells with a RUNX1-ETO expression plasmid resulted in expression of the RUNX1-ETO protein in these cells, which is also detected endogenously in the Kasumi-1 cell line that contains the t(8;21) translocation (Fig. 2D). In contrast to RUNX1, overexpression of RUNX1-ETO in K562 cells failed to increase ITGB4 promoter reporter activity (Fig. 2C), in

keeping with its commonly described role as a transcriptional repressor. To determine whether RUNX1-ETO can however, compete with RUNX1 activation of the ITGB4 promoter, K562 cells were transfected with the -1199/+144 ITGB4 reporter, RUNX1 expression plasmid, and increasing amounts of RUNX1-ETO expression plasmid. RUNX1-ETO repressed RUNX1 activation of the ITGB4 reporter in a dose-dependent manner (Fig. 2E). These results therefore suggest that while endogenous ITGB4 expression is higher in cells in which RUNX1-ETO is expressed (Fig. 1B, 1C), RUNX1 activates the ITGB4 promoter, while RUNX1-ETO can compete with RUNX1, inhibiting RUNX1 activation of the promoter when it is examined in isolation in reporter assays.

To determine whether RUNX1 also regulates the ITGA6 gene, the ITGA6 promoter reporter (-675/+242 ITGA6) was transfected into K562 cells. As for the ITGB4 promoter reporter, basal activity of the ITGA6 promoter reporter was detected and this activity increased approximately 14-fold upon co-transfection with the RUNX1 expression construct (Fig. 2F). Further, co-transfection with a RUNX1-ETO expression construct repressed basal reporter activity approximately 0.5 fold (Fig. 2F).

Put together, these data suggest that RUNX1 regulates both the ITGB4 and ITGA6 promoters.

RUNX1 activates the ITGA6 promoter through a consensus RUNX1 binding motif

To determine the site at which RUNX1 regulates the ITGA6 promoter, a deletion construct (-142/+242 ITGA6) was created, which deleted 6 of the 9 potential RUNX1 binding motifs (Fig. 3A). K562 cells were transfected with either the -675/+242 ITGA6 construct or the deletion construct, along with the RUNX1 expression plasmid. As before, overexpression of

RUNX1 in K562 cells increased activity of the -675/+242 ITGA6 reporter (Fig. 3B). However, for the deletion construct this effect was significantly reduced (Fig. 3B). These data therefore suggest that RUNX1 is partly acting through a RUNX1 responsive element located within the deleted region of the promoter, between -675 bp to -141 bp upstream of the transcription start site.

Further analysis of the ITGA6 promoter sequence determined that there were 6 putative RUNX1 binding sites located within the RUNX1 responsive region but only one of these perfectly matches the RUNX1 consensus sequence (ACCACA at -237 bp to -232 bp). To determine if RUNX1 activates the ITGA6 reporter through this site, a reporter construct was generated in which this site was mutated (-675/+242*mut* ITGA6). K562 cells were again transfected with the -675/+242 ITGA6 reporter, the deletion reporter or the mutant reporter, with or without the RUNX1 expression plasmid. As before, RUNX1 activated the full-length ITGA6 reporter approximately 10 fold, and this was significantly reduced (to approximately 5 fold) when either the region from -675 bp to -141 bp was deleted or the RUNX1 consensus sequence was mutated (Fig. 3C). These data suggest that RUNX1 can activate the ITGA6 promoter through a consensus sequence at -237 bp to -232 bp (within the region -675 bp to -141 bp), which contributes about half of the RUNX1 response of the promoter. The remainder of the RUNX1 response of the promoter is mediated through the region from -141 to +242, and/or the plasmid backbone which has previously been shown to contribute low level response to RUNX1 through cryptic RUNX1 sites (Oakford et al., 2010).

ChIP assays were then used to examine RUNX1 binding at the ITGA6 promoter. DNA immunoprecipitated with RUNX1 antibodies was amplified by quantitative PCR, using primers adjacent to the transcription start site. In KG-1a cells, RUNX1 was significantly

enriched at the ITGA6 promoter compared to the rhodopsin promoter, which is silenced in hematopoietic cells and therefore a negative control for RUNX1 binding (Fig. 3D). Similarly, RUNX1 binding was detected at the ITGA6 promoter in Kasumi-1 cells, but was undetectable at the rhodopsin promoter (Fig. 3E). Finally, ChIP analysis using an ETO antibody, showed enrichment at the ITGA6 promoter in Kasumi-1 cells compared to the rhodopsin promoter, suggesting binding of RUNX1-ETO, although this was not statistically significant (Fig. 3F). In support of these data, RUNX1 binding was detected at the ITGA6 promoter in both SKNO-1 cells and Kasumi-1 cells, in previously published ChIP-seq data (co-ordinates chr2: 173291994 – 173293230 and chr2: 173291756 – 173292615, respectively; (Martens et al., 2012)). RUNX1-ETO binding was also detected at the ITGA6 promoter in Kasumi-1 cells in this dataset.

Together, these data suggest that RUNX1 binds to the ITGA6 promoter and regulates promoter activity, including through a consensus motif in the promoter located at -237 bp to -232 bp.

RUNX1 activates the ITGB4 promoter in the absence of a RUNX1 consensus binding motif

To determine the region through which RUNX1 activates the ITGB4 promoter, deletion promoter constructs were analyzed in reporter assays. Deletion constructs were generated from the -1199 bp to +144 bp ITGB4 promoter reporter construct that was found to be RUNX1 responsive, sequentially removing the 5' region of the promoter (Fig. 4A). This region of the ITGB4 promoter contains 7 potential RUNX1 binding motifs, 4 between -700 and -1199 bp, and 3 between -400 and -100 bp, although none of these are a 100% match to the RUNX1 consensus sequence. The series of deletion constructs were transfected into K562

cells, either with or without a RUNX1 expression construct, and reporter activity measured. These assays determined that the constructs containing the regions from -758 bp to +144 bp and also -295 to +144 retained RUNX1 responsiveness (Fig. 4B). However, deletion of the region from -295 to -58 bp upstream of the promoter resulted in a significant reduction in the response to RUNX1 overexpression (-57/+144 ITGB4, Fig. 4B). While this reporter construct retained some responsiveness to RUNX1, this was almost completely abolished by deletion of the region downstream of the transcription start site (-57/+13; Fig. 4B). These data suggest that a RUNX1 responsive region of the ITGB4 promoter is located in the region from -295 bp to -58 bp upstream of the transcription start site. To test this further a construct was generated which contained the promoter region from -758 bp to +144 bp, but deleted the region from -295 bp to -58 bp (-758/+144 Δ-295/-58; Fig. 4A,C). These promoter reporter constructs were then transfected into K562 cells, either with or without a RUNX1 expression construct. Specific deletion of the region from -295 to -58 resulted in a significant decrease in RUNX1 activation of the promoter (Fig. 4C), to the same levels as observed by deletion of the entire region from -1199 to -58 (Fig. 4B), therefore confirming that the region from -295 to -58 is required for RUNX1 activation of the ITGB4 promoter.

The region of the ITGB4 promoter from -295 to -58 contains two potential RUNX1 binding motifs. Therefore to narrow down the RUNX1 responsive region further, another deletion construct was generated to delete one of these motifs (-176/+144 ITGB4; Fig. 4A,D). While deletion of the region from -295/-58 (-57/+144 ITGB4) again demonstrated reduction in RUNX1 responsiveness, this was not observed following deletion of the region from -295 bp to -176 bp (-176/+144 ITGB4) and thus removing the motif at -196 bp to -201 bp. These results therefore suggest that this site is not responsible for RUNX1 activation of the promoter and that this activity is located with -58 bp to -175 bp of the ITGB4 promoter. This

region contains one putative RUNX1 binding motif (GCCGCA), which while not a 100% match to the consensus sequence has been found to bind Runx1 in a previous study in mouse (Tanaka et al., 2012). To determine if RUNX1 activates the ITGB4 promoter through this site, a reporter construct was generated in which this site was mutated (-295/+144mut ITGB4; Fig. 4A,E). As before, while removal of this entire region reduced RUNX1 responsiveness of the promoter (-57/+144 ITGB4), mutation of the potential RUNX1 binding site did not affect the ability of RUNX1 to activate the promoter. Put together, these data suggest that RUNX1 can activate the ITGB4 promoter through the region from -175 to -58, but does not do so through a recognized RUNX1 consensus sequence.

ChIP assays were then used to examine RUNX1 binding to the ITGB4 promoter. DNA immunoprecipitated with RUNX1 antibodies was amplified by quantitative PCR using primers adjacent to the transcription start site. RUNX1 was enriched at the ITGB4 promoter, compared to the rhodopsin promoter, in KG-1a cells (Fig. 4F). Further, RUNX1 enrichment was observed at the ITGB4, but not rhodopsin promoter in Kasumi-1 cells (Fig. 4G). However, ChIP analysis using an ETO antibody failed to detect enrichment at the ITGB4 promoter compared to the rhodopsin promoter (data not shown), suggesting RUNX1-ETO is not specifically enriched at the ITGB4 promoter in Kasumi-1 cells. These data are in keeping with publicly available ChIP-seq analysis in which RUNX1 but not RUNX1-ETO binding was detected at the ITGB4 promoter in both SKNO-1 and Kasumi-1 cells (co-ordinates chr17:73716850-73717900; (Martens et al., 2012)).

Together, these data suggest that RUNX1 is recruited to and can activate the ITGB4 promoter through a mechanism that does not require a consensus RUNX1 sequence motif. Interestingly

however, the available data failed to detect RUNX1-ETO binding to the endogenous ITGB4 promoter.

Regulation of the ITGB4 promoter by a distal regulatory element

Given that many promoters are subject to complex regulatory control involving distal regulatory elements, we next investigated the possibility that a RUNX1 binding motif for ITGB4 was located distal from the promoter. To determine whether the ITGB4 promoter interacts with other regions of the genome, ChIA-PET data from the ENCODE project (ENCODE 2012) were interrogated. Specifically, analysis of RNA Polymerase II ChIA-PET data produced in K562 cells (GSM970213) identified three interactions involving the ITGB4 promoter. These are a shorter range interaction with a region located -1,805 bp to -3,459 bp upstream of the transcription start site; a longer range interaction with a region located +8491 bp to +10,343 bp downstream; and another longer range interaction with a region located -12,053 bp to -14,003 bp upstream (Fig. 5A,B). While these interactions are relatively weak, this may reflect the low levels of ITGB4 expression in K562 cells. No such interactions were observed at the ITGA6 promoter (data not shown). To determine whether any of the regions interacting with the ITGB4 promoter represent putative enhancer elements, the histone modification data from the ENCODE project were analyzed for H3K4me1 and H3K27ac histone modifications representative of enhancer regions (Rada-Iglesias et al., 2011; Taberlay et al., 2011). Only one of these regions, located -12,053 bp to -14,003 bp upstream of the ITGB4 transcription start site displayed features suggestive of an enhancer element, with the presence of both H3K4me1 H3K27Ac, as well as a DNase I hypersensitive site, towards the 5' end of this region (Fig. 5A). Further, analysis of publicly available ChIP-seq data revealed RUNX1 binding to the 5' region in both CD34+ and Kasumi-1 cells (Beck et al., 2013; Ptasinska et al., 2014). In support of this ChIP analysis for RUNX1 binding in KG-1a cells

detected enrichment of RUNX1 at the ITGB4 promoter and the 5' end of the distal interacting region (Fig. 5C). In contrast, little or no enrichment for RUNX1 was observed at the 3' end of the distal interacting region, or the Rhodopsin promoter.

To determine whether this distal interacting region can function as an enhancer together with the ITGB4 promoter, the 5' end of this region encompassing the DH site (-13,845 bp to -14,156 bp; enh-1), and the 3' end (-12,077 bp to -12,408 bp; enh-2), were cloned adjacent to the minimal RUNX1 responsive promoter region (-176/+144 ITGB4; Fig. 5B). These constructs were transfected into K562 cells and luciferase activity measured after 24 hours. The enh-1 region demonstrated regulatory activity with a significant increase in reporter activity compared to the promoter region alone (Fig. 5D). In contrast, the enh-2 construct showed no change in reporter activity compared to the promoter region alone (Fig. 5D). In order to determine the capacity for RUNX1 to activate the distal regulatory element (enh-1), K562 cells were transfected with the constructs with or without the RUNX1 expression plasmid. Fold change in activity of each construct was analyzed after 24 hours. As described previously, RUNX1 activated the minimal promoter construct (-176/+144 ITGB4; Fig. 5E), however unexpectedly, reporter activity was not further increased in the presence of the distal regulatory element (-176/+144 enh-1 ITGB4; Fig. 5E).

Finally, to explore the regulation of the endogenous ITGB4 and ITGA6 genes by RUNX1, stable clonal cell lines overexpressing RUNX1 were generated in K562 cells. A two fold increase in RUNX1 expression was achieved, as demonstrated by quantitative PCR analysis of clonal lines (Fig. 5F). While this resulted in an approximately two fold increase in *ITGA6* expression (Fig. 5G), no difference in *ITGB4* expression was observed between the control and RUNX1 overexpressing cells.

Put together, these data suggest that RUNX1 regulates the ITGA6 gene through a consensus binding site in the ITGA6 promoter. In contrast, while RUNX1 can bind to both the ITGB4 promoter and an interacting upstream region, and can activate the promoter in isolation, its regulation of the ITGB4 gene is complex and may require interacting partners.

Discussion

RUNX1 is commonly described as a sequence-specific DNA binding transcription factor, which binds to the promoters of its target genes and regulates their transcriptional activity (Meyers et al., 1993). However, it is evident now that RUNX1 regulation of gene expression is more complex than this, encompassing multiple regulatory layers involving interaction with other co-factors or transcription factors, distal regulatory elements and epigenetic factors (Bowers et al., 2010; Elagib et al., 2003; Huang et al., 2009; Reed-Inderbitzin et al., 2006). Here we provide evidence that the transcription factor RUNX1 contributes to the regulation of both the ITGA6 and ITGB4 genes, which encode the α6β4 integrin receptor, although it functions at these genes through distinct mechanisms.

ChIP assays and reporter analysis determined the region of the ITGA6 and ITGB4 promoters through which RUNX1 functions. These data suggest that RUNX1 binds to the ITGA6 promoter via a consensus RUNX1 binding motif located -237 bp to -232 bp upstream of its transcription start site. Meanwhile, binding to the ITGB4 promoter appears to be via an indirect mechanism with RUNX1 shown to activate the isolated ITGB4 promoter in the absence of a consensus binding motif. The RUNX1 responsive region of the ITGB4 promoter was located -175 bp to -58 bp upstream of the transcription start site. This region contained a potential RUNX1 binding motif however mutation of this site failed to repress activation of

the ITGB4 promoter. This demonstrated that while RUNX1 activates the ITGB4 promoter in isolation, binding is not via a recognized RUNX1 consensus binding motif and suggests an interacting partner may be involved in recruitment. ChIP analysis confirmed RUNX1 occupancy at the ITGB4 promoter, however binding *via* a distal regulatory region could not be ruled out, given that DNA looping and chromatin interactions with the promoter were detected in ChIA-PET data. Indeed, the ITGB4 promoter interacts with an upstream regulatory region, approximately -13,957 bp to -14,020 bp upstream of the transcription start site, and this region enhanced ITGB4 promoter activity in reporter assays. Further, RUNX1 occupancy of this upstream regulatory region was demonstrated, although like the promoter, this was in the absence of a recognized RUNX1 binding motif. While reporter assays failed to detect increased RUNX1 responsiveness of the promoter in combination with the upstream regulatory region, this may be attributed to the region being cloned directly adjacent to the ITGB4 promoter, and unable to establish the appropriate environment to facilitate promoter-enhancer interactions.

RUNX1 is a relatively weak transcription factor on its own and therefore often regulates target genes through multi-protein complexes assembled at promoter and enhancer regions. In recent genome-wide studies, RUNX1 was found to frequently co-occupy regions of the genome with other transcription factors such as SCL, LYL1, LMO2, ERG, FLI1, GATA1 and GATA2 (Beck et al., 2013; Tijssen et al., 2011; Wilson et al., 2010)). Interestingly, the majority of these regions lack RUNX1 consensus binding motifs. The lack of RUNX1 consensus motifs despite RUNX1 occupancy of the DNA suggests that RUNX1 is recruited indirectly to these regions, *via* interactions with other transcription factors. Further, motif analysis of the RUNX1 responsive region of the ITGB4 promoter and the interacting distal regulatory region identified potential binding sites for many of these transcription factors,

suggesting that RUNX1 may be recruited to the ITGB4 promoter *via* such a multi-transcription factor complex. Further analysis of these regions is required to determine the RUNX1 complex that may assemble at the promoter and whether assembly of this complex is facilitated by interactions with the distal regulatory region. An absence of such factors may explain the lack of effect of overexpression of RUNX1 on expression of the endogenous ITGB4 gene. Alternatively, this may suggest that the RUNX1 protein detected at the ITGB4 promoter is not acting as a traditional transcriptional activator but is contributing other functions such as complex scaffolding. This would be in keeping with a recent study which suggested an architectural role for RUNX1 following genome-wide analysis of RUNX1 binding in breast cancer cells (Barutcu et al., 2016).

The ITGA6 and ITGB4 genes encode the integrin α6β4, a laminin receptor that plays an integral role in hemidesmosome organization. Furthermore, this integrin receptor plays a role in cell migration, and signaling through α6β4 has be shown to stimulate oncogenic pathways including p53 and PI3K signaling (reviewed in (Stewart and O'Connor, 2015)). These two integrin subunits promote metastatic potential and are frequently disrupted in a variety of solid tumors (Lu et al., 2008; Stewart et al., 2016). In leukemia, where RUNX1 action is often disrupted, dysregulated expression of RUNX1 target genes is evident (Ichikawa et al., 2006). Moving forward from our findings it will be imperative to determine precisely how disruption of RUNX1 affects regulation of these two integrin genes. Interestingly, while RUNX1-ETO inhibited RUNX1 activation of both the ITGA6 and ITGB4 promoters in reporter assays, analysis of available gene expression data from cells containing the t8;21 translocation (Fig. 1C,D) suggests that endogenous *ITGA6* expression is not altered in the presence of the RUNX1-ETO protein, while *ITGB4* expression is increased. While RUNX1-ETO has generally been demonstrated to functionally compete with RUNX1 to inhibit gene

expression, it has also been found to increase gene expression in some circumstances (DeKelver et al., 2013). In addition, the variant RUNX1-ETO9a transcript, which is detected in Kasumi-1 cells and in 27 of 37 individuals with t(8;21) AML in a previous study, has been found to act as an activator due to the absence of a C-terminal inhibitory domain (Yan et al., 2004; Yan et al., 2006). Interestingly though, RUNX1-ETO binding at the ITGB4 promoter was not detected at the promoter in this study, nor in previous genome-wide analysis (Martens et al., 2012), suggesting that the effect of RUNX1-ETO on the ITGB4 promoter may be indirect. However, it is also plausible that this could be an effect of alterations in chromatin interactions and organization due to reduced RUNX1 expression in cells containing the t(8;21) translocation, and this warrants further investigation. The data presented here serves to further emphasize the complexity of regulation of gene expression in the endogenous context, where the competing actions of RUNX1 and RUNX1-ETO, as well as the influence of distal regulatory regions and chromatin environment are at play.

In terms of the implications of altered α6β4 expression in hematopoietic cells, future work will need to determine the levels of this integrin receptor on the surface of cancer cells relative to normal cells, how signaling through the integrin pathway is altered and consequent functional implications for disease progression. It should also be noted that integrin α6β4 has been shown to regulate the expression of approximately 500 genes (Chen et al., 2009) and numerous miRNAs (Gerson et al., 2012). In regulating the levels of these two cell adhesion proteins, RUNX1 may indirectly regulate cell-matrix or cell-cell adhesion contacts as well as signaling and transcriptional programs of the cell. As myeloid cells have inherent cell motility through the vasculature, the implications from dysregulated integrin expression in AML may include a role for RUNX1 in dysregulating signaling and subsequent gene expression patterns via the α6β4 receptor.

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Figure Legends

Fig. 1. Expression levels of RUNX1, RUNX1-ETO, ITGB4 and ITGA6 in leukemic cells and cell lines. (A, B) RNA was isolated from K562, KG-1a and Kasumi cells, reverse transcribed and levels of RUNX1 and RUNX1-ETO (A) or ITGB4 and ITGA6 (B) measured using qPCR. Copy number was normalized to GAPDH. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using one-way ANOVA, Newman-Keuls Multiple Comparison Test, *P<0.05, ***P<0.001, ns P>0.05. (C, D) Expression levels of ITGB4 (C) and ITGA6 (D) in patient-derived primary leukemic cells was determined based on publicly available microarray data (Hebestreit et al., 2012). Statistical significance was determined using Students' t Test ***P<0.001.

Fig. 2. RUNX1 activates the ITGB4 and ITGA6 promoters. A: Schematic of the ITGB4 and ITGA6 promoter regions used in reporter assays. Scale indicates bp relative to the transcription start site (arrow). White boxes represent putative RUNX1 binding motifs, grey box represents a RUNX1 consensus binding motif. B: K562 cells were transfected with or without a RUNX1 expression plasmid, nuclear extracts were prepared and RUNX1 and histone H3 detected by western blotting. C: K562 cells were transfected with the ITGB4 promoter reporter construct, along with either the RUNX1 or RUNX1-ETO expression vector, cell lysate extracted 24 hours post-transfection and luciferase reporter activity measured. D: Protein was extracted from K562 cells transfected with a RUNX1-ETO expression plasmid, along with non-transfected Kasumi-1, K562 and KG-1a cells. Nuclear extracts were prepared and levels of RUNX1-ETO were detected via western blot. E: K562 cells were transfected with the ITGB4 promoter reporter, RUNX1 expression plasmid, and increasing amounts of RUNX1-ETO expression plasmid. Protein was harvested 24 hours post-transfection and fold change in luciferase activity measured. F: K562 cells were transfected with the ITGA6 promoter reporter, along with either the RUNX1 or RUNX1-ETO expression vector, protein extracted 24 hours post-transfection and luciferase activity measured. In C-D, values are expressed as mean ±SEM (n=3). Statistical significance was determined using Students' t Test, *P<0.05, ***P<0.001.

Fig. 3. RUNX1 activates the ITGA6 promoter via a consensus binding motif. A: Schematic of the ITGA6 promoter deletion and mutant constructs used in reporter assays. Scale indicates bp, relative to the transcription start site (arrow). White boxes represent putative RUNX1 binding motifs, grey box represents a RUNX1 consensus binding motif. B-C: K562 cells were transfected with the promoter reporter constructs as indicated, either with or without RUNX1 expression plasmid. Protein was isolated from transfected cells after 24 hours and fold change in luciferase activity in the presence of RUNX1 determined. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using Student's t Test, **P<0.01, ***P<0.001. D-E: ChIP assays were performed with anti-RUNX1 antibody in KG-1a (D) and Kasumi-1 (E) cells. Immunoprecipitated DNA was amplified using ITGA6 and Rhodopsin promoter primers, as indicated. The data are presented as a percentage of total input DNA. Values are expressed as mean ±SEM (n=3-6). Statistical significance was

determined using Student's t Test, ***P<0.001. F: ChIP assays were performed with anti-RUNX1-ETO antibody in Kasumi-1 cells. Immunoprecipitated DNA was analyzed as in (D).

Fig. 4. RUNX1 activates the ITGB4 promoter in the absence of a consensus binding motif. A: Schematic of the ITGB4 promoter constructs used in reporter assays. Scale indicates bp relative to the transcription start site (arrow). White boxes represent putative RUNX1 binding motifs. B-E: K562 cells were transfected with the indicated ITGB4 promoter reporter constructs either with or without the RUNX1 expression plasmid. Protein was isolated from cells 24 hours post-transfection and fold change in luciferase activity in the presence of RUNX1 determined. Values are expressed as mean ±SEM (n=3). Statistical significance was determine using one-way ANOVA, Newman Keuls Multiple Comparison Test, ***P<0.001, **P<0.01, *P<0.05. F-G: ChIP assays were performed with anti-RUNX1 antibody in KG-1a (F) and Kasumi-1 (G) cells. Immunoprecipitated DNA was amplified using ITGB4 and Rhodopsin promoter primers, as indicated. The data are presented as a percentage of total input DNA. Values are expressed as mean ±SEM (n=3-6).

Fig. 5. ITGB4 promoter activity is enhanced by a distal regulatory region. A: Screen shot of ChIA-PET, histone CHIP-seq and DNAse I hypersensitive site (DHS) data from the ENCODE project (ENCODE 2012), visualized in the UCSC genome browser. ChIA-PET and ChIP-seq data were analyzed to identify putative enhancer regulatory elements of the ITGB4 promoter. Three regions of DNA found to interact with the ITGB4 promoter through RNA polymerase II in K562 cells were identified as possible enhancers. The DNA loops of the three interacting regions of DNA are shown as dark grey bars. Histone ChIP-seq data of histone marks characteristic of enhancer and promoter regions (H3K4me3, H3K4me1 and H3K27ac) are shown, DNase hypersensitive signal is shown as a black wiggle plot, with DHS indicated by black boxes. The ITGB4 promoter and putative enhancer regions are highlighted in the grey boxes. B: Schematic of the ITGB4 promoter and putative enhancer constructs used in reporter assays. Scale indicates bp relative to the transcription start site (arrow). C: ChIP assays were performed with anti-RUNX1 antibody in KG-1a cells. Immunoprecipitated DNA was amplified using the indicated primers, as indicated. The data are presented as a percentage of total input DNA. Values are expressed as mean ±SEM (n=3-6). D: K562 cells were transfected with the indicated ITGB4 reporter constructs. Protein was isolated from cells 24 hours post-transfection and luciferase activity measured. Values are expressed as mean ±SEM (n=3). Statistical significance was determined using one-way ANOVA, Newman Keuls Multiple Comparison Test, *P<0.05. E: K562 cells were transfected with the indicated ITGB4 promoter reporter constructs either with or without the RUNX1 expression plasmid. Protein was isolated from cells 24 hours post-transfection and fold change in luciferase activity in the presence of RUNX1 determined. Values are expressed as mean ±SEM (n=3). F-H: K562 cells were transfected with either pTracer-RUNX1 or pTracer-CMV/BSd (control) plasmid and clones selected. RNA was isolated from 5 control and 4 RUNX1 expressing clones, reverse transcribed and levels of RUNX1 (F), ITGB4 (G) and ITGA6 (H) measured using qPCR. Copy number was normalized to GAPDH. Values are expressed as mean ±SEM (n=4 or 5). Statistical significance was determined using Students t Test, **P<0.01, *P<0.05.

Table 1: PCR primers used to generate reporter constructs

Reporter construct	Primer Sequences [a]
-1199/+144 ITGB4	5’ – TGCTA*CTCGAG*GCATGGTTTGGACAGTGCT – 3’
	5’ – TGCTA*AAGCTT*CCCGTCCTGGACCTACCT – 3’
-675/+242 ITGA6	5’ – TGCTA*CTCGAG*CATCCTTGACTTGCGTGACT – 3’
	5’ – TGCTA*AAGCTT*CGACAGGTAGAGCAAGCACA – 3’
-758/+144 ITGB4	5’ – TGCTA*CTCGAG*CTGCTCTCAGAGGACTGACG – 3’
	5’ – TGCTA*AAGCTT*CCCGTCCTGGACCTACCT – 3’
-295/+144 ITGB4	5’ – TGCTA*CTCGAG*CTGCTCTCAGAGGACTGACG – 3’
	5’ – TGCTA*AAGCTT*CCCGTCCTGGACCTACCT – 3’
-57/+144 ITGB4	5’ – TGCTA*CTCGAG*ATGCAGCCGGTCTGACTC – 3’
	5’ – TGCTA*AAGCTT*CCCGTCCTGGACCTACCT – 3’
-57/+13 ITGB4	5’ – TGCTA*CTCGAG*ATGCAGCCGGTCTGACTC – 3’
	5’ – TGCTC*AAGCTT*AGGCGGGCAGCGCTTTAT – 3’
-758/+144 Δ-295/-58 ITGB4	5’ – TGCTA*CTCGAG*CTGCTCTCAGAGGACTGACG – 3’
	5’ – TGCTA*AAGCTT*CCCGTCCTGGACCTACCT – 3’
	5’ – TGCTA*CCCGGG*ATGCAGCCGGTCTGACTC – 3’
	5’ – TGCTA*AAGCTT*CCCGTCCTGGACCTACCT – 3’
-176/+144 ITGB4	5’ – TGCTA*CTCGAG*CTAGCCGATCGGGGCGCT – 3’
	5’ – TGCTA*AAGCTT*CCCGTCCTGGACCTACCT – 3’
-295/+144*mut* ITGB4	5’ – ATTAA*CCCGGG*GCAGTCCGCGCA – 3’
	5’ – ATTAA*AGATCT*CCCGCGGCGCCCGCCCA – 3’
	5’ – ATTAA*AGATCT*AGCCCTTTCCGGGGGGCGG – 3’
	5’ – TGCTA*AAGCTT*CCCGTCCTGGACCTACCT – 3’
-176/+144 enh-1 ITGB4	5’ – TGCTA*GGATCC*GAGGCGGCAGCTCATTGT – 3’
	5’ – TGCTA*CTCGAG*GTGCCATTTCAGACCACCT – 3’
-176/+144 enh-2 ITGB4	5’ – TGCTA*GGATCC*CTAGGGCTCGATTTCCAAAG – 3’
	5’ – TGCTA*CTCGAG*CCTCCTGAGTAGCTGGGAAT – 3’
-142/+242 ITGA6	5’ – TGCTA*CTCGAG*CAGCTGGAGACGCCAGAG – 3’

	5’ – TGCTA*AAGCTT*CGACAGGTAGAGCAAGCACA – 3’
-675/+242*mut* ITGA6	5’ – TGCTA*CTGCAG*CATCCTTGACTTGCGTGACT – 3’
	5’ – TGCTA*GATATC*TGCCGAGTAGCACAGAGCGA – 3’
	5’ – TGCTA*GATATC*ATTCTGTCCACAGAGGGCGG – 3’
	5’ – TGCTA*AAGCTT*CGACAGGTAGAGCAAGCACA – 3’

[a]Restriction enzyme sites within primers are italicized

Table 2: PCR primers used for expression and ChIP analysis

Gene / Region	Primer Sequence
RUNX1[a]	For: 5' – CACCTACCACAGAGCCATCA – 3'
	Rev: 5' – CTCGGAAAAGGACAAGCTCC – 3'
RUNX1-ETO[a]	For: 5' – AATCACAGTGGATGGGCCC – 3'
	Rev: 5' – TGCGTCTTCACATCCACAGG – 3'
ITGB4[a]	For: 5' – TTAAGAGAGCCGAGGAGGTG – 3'
	Rev: 5' – GGCAGTCCTTCTTCTTGTGC – 3'
ITGA6[a]	For: 5' – CCAAAAATTACTTTGGGGCTAA – 3'
	Rev: 5' – TCAGCTTTCATATCTATTCAGTCTCTG – 3'
GAPDH[a]	For: 5' – AAATATGATGACATCAAGAAGG – 3'
	Rev: 5' – AGCCCAGGATGCCCTTGAGGG – 3'
ITGB4 Promoter[b]	For: 5' – CTCGGACAGTCCCTGCTC – 3'
	Rev: 5' – GCTGCCGCTAGGAGATGG – 3'
ITGA6 Promoter[b]	For: 5' – GCGTCCTCGTCACTTGATAA – 3'
	Rev: 5' – AATGAGCCCGTTGTTCTCTG – 3'
ITGB4 enh-1[b]	For: 5' – TGAAACGGGTTTCCCAGAC – 3'
	Rev: 5' – ATCGCCAAAGATCATGAAGG – 3'
ITGB4 enh-2[b]	For: 5' – GCTATTGAGCCTGGTGCAGT – 3'
	Rev: 5' – CCTCCTGAGTAGCTGGGAAT – 3'
Rhodopsin Promoter[b]	For: 5' – CCAATCTCCCAGATGCTGAT – 3'
	Rev: 5' – TAAAGTGACCTCCCCCTCCT – 3'

[a]Expression analysis, [b]ChIP analysis

Fig. 1

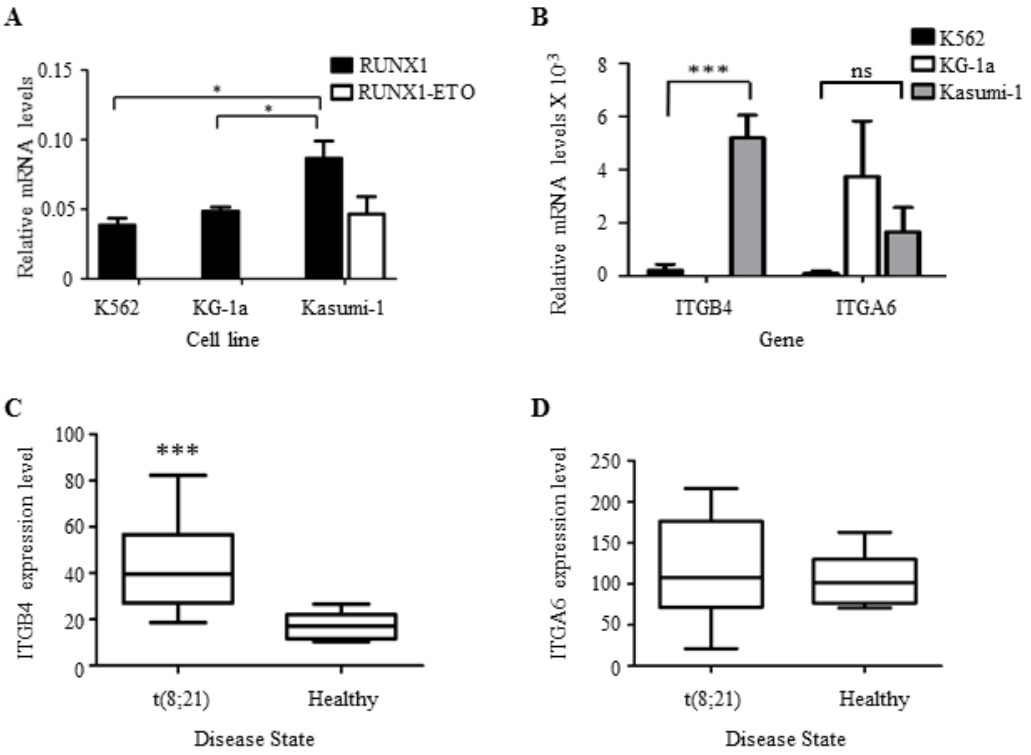

Fig. 2

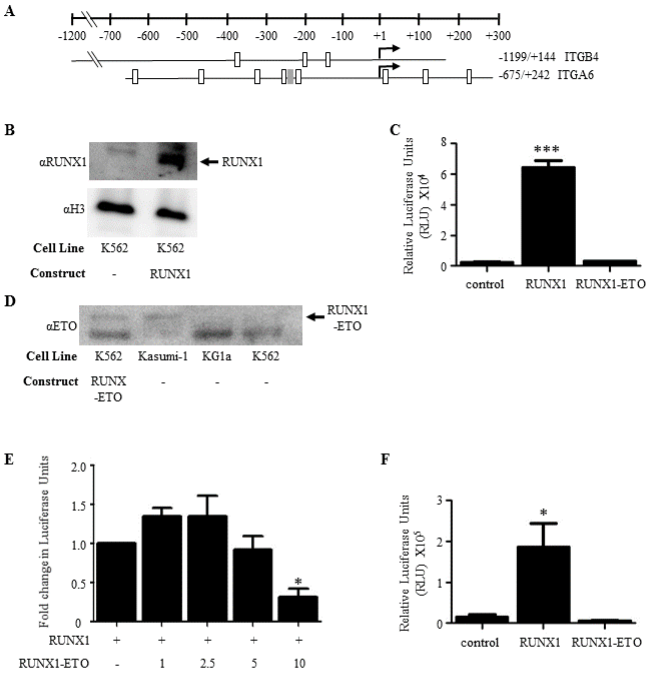

Fig. 3

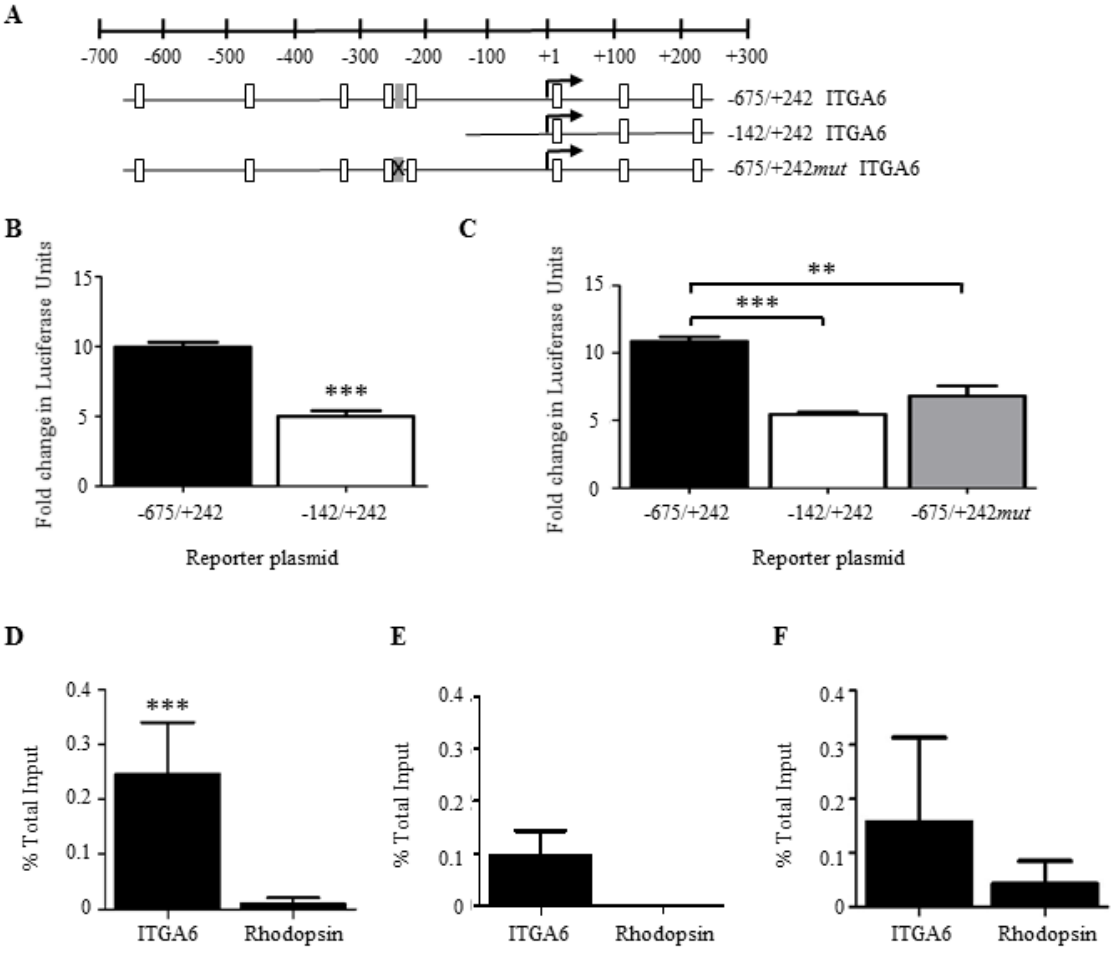

Fig. 4

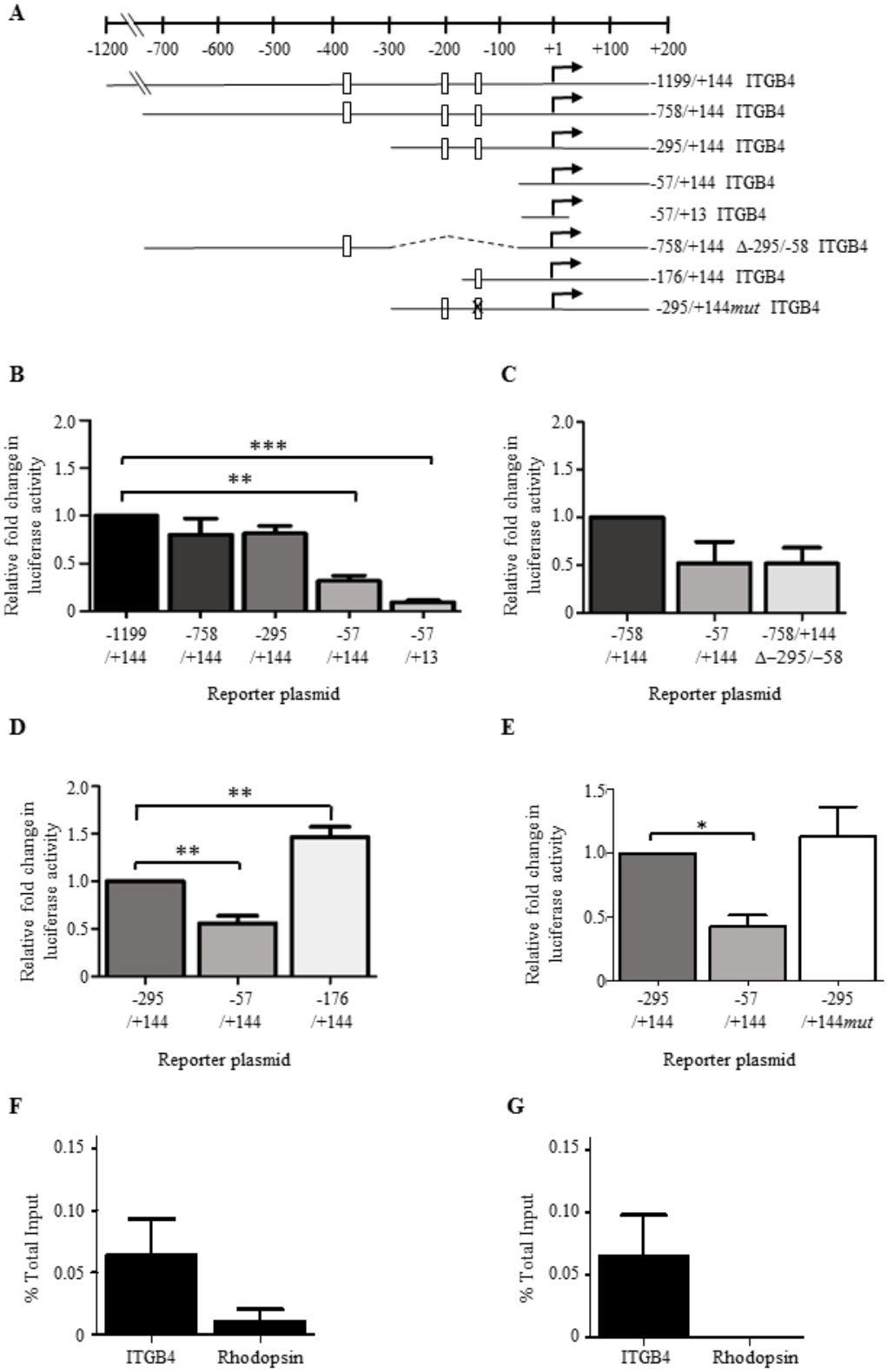

Fig. 5