The adaptor protein ARA55 and the nuclear kinase HIPK1 assist c-Myb in recruiting p300 to chromatin

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Abstract

LIM-domain proteins, containing multiple cysteine-rich zinc finger-like motifs, have been shown to play diverse roles in several cellular processes. A common theme is that they mediate important protein-protein interactions that are key to their function. Androgen receptor-associated protein 55 (ARA55) belongs to this family of bridging proteins containing four C-terminal LIM domains. It has a dual role with functions both at focal adhesions and in the nucleus, apparently shuttling between the two compartments. In the present work, we have expanded our understanding of its nuclear functions by showing that it interacts with three nuclear regulators not previously linked to ARA55. We first identified ARA55 as a novel interaction partner of the nuclear kinase HIPK1 and found that ARA55, like HIPK1, also interacts with the transcription factor c-Myb. In search of a function for these associations, we observed that the coactivator p300 not only binds to c-Myb, but to ARA55 as well. When combined, c-Myb, p300, HIPK1 and ARA55 caused strong synergistic activation of a chromatinized reporter gene. In parallel, all partners, including p300, were efficiently recruited to chromatin at the c-Myb-bound promoter. Consistent with this cooperation, we found that c-Myb and ARA55 share a common set of target genes in an osteosarcoma cellular context. We propose that ARA55 and HIPK1 assist c-Myb in recruiting the coactivator and acetyltransferase p300 to chromatin.

Keywords

Transcription factor; c-Myb; HIPK1; ARA55; Hic-5; TGFB1I1; p300; coactivator.

Abbreviations

The abbreviations used are:

2KR, double mutant of c-Myb (K503R+K527R); **AR**, androgen receptor; **ARA55**, AR coactivator with a molecular mass of 55 kDa; **c-Myb**, v-myb avian myeloblastosis viral oncogene homolog; **ChIP**, chromatin immunoprecipitation; **CoIP**, co-immunoprecipitation; **DBD**, DNA-binding domain; **GST**, Glutathione S-transferase; **HAT**, Histone acetyltransferase; **hcM**, human c-Myb; **HIPK1**, homeodomain interacting protein kinase 1; **KIX**, the CREB and c-Myb interaction domain of p300; **LIM**, a cysteine-rich zinc finger-like motifs named after its initial discovery in the proteins **L**in11, **I**sl-1 & **M**ec-3; **p300**, E1A binding protein p300 or histone acetyltransferase p300; **PAGE**, Polyacrylamide gel electrophoresis; **SIM**, SUMO-interaction motifs; **SUMO**, small ubiquitin-related modifier; **TAD**, transactivation domain; **TF**, transcription factor

1. Introduction

TGFB1I1/ARA55/HIC-5 is a LIM-domain protein and a member of the paxillin family, implicated in several different processes in the cell, such as cell growth, proliferation, migration, differentiation and senescence, with a defined role in focal adhesion, Wnt and TGFβ signalling, and nuclear receptor activation [1-3]. This variety of functions is reflected in several designations of the gene, TGFB1I1 (Transforming Growth Factor Beta 1 Induced Transcript 1), ARA55 (Androgen Receptor-Associated Protein Of 55 kDa; preferred designation in this work), or HIC-5 (Hydrogen Peroxide-Inducible Clone 5 Protein). The many functions are explained by the protein operating as a molecular adapter, coordinating multiple protein-protein interactions. An important feature of the gene is its response to TGFβ and oxidative stress. Since the protein has functions both at the cell membrane and in the nucleus, it may convey signals between these compartments [4].

While the cytoplasmic role of ARA55 relates to it functioning as a molecular scaffold at focal adhesions [1,2,5-7], its nuclear functions are linked to ARA55 operating as a coactivator, in particular towards nuclear receptors regulating glucocorticoid, androgen, and progesterone-responsive gene programs [3,8-12]. Inactivation of ARA55 resulted in reduced androgen receptor (AR) activity in prostate cancer cell lines [3,9,13]. Conversely, aging was found to up-regulate ARA55 in stromal cells, inducing androgen-mediated prostate cancer cell proliferation and migration [14]. The adaptor protein shuttles between the cytosolic (focal adhesion complexes) and the nuclear compartments, with TGFβ and oxidative stress promoting its nuclear localization by inhibiting its nuclear export [2]. Once in the nucleus, ARA55 functions as a transcriptional co-activator for AR and other specific transcription factors (TFs), such as nuclear receptors, SMADs and Sp1 [3,12,15].

ARA55 is linked to cancer development in several ways. ARA55 expression becomes induced by TGFβ, contributing to epithelial mesenchymal transition (EMT), cell migration, and invasion [16,17]. Furthermore, ectopic expression of ARA55 is sufficient to promote normal human breast epithelial cells to undergo EMT [16]. Both Paxillin and ARA55 impact the canonical metastasis cascade at several levels through their ability to coordinate plasticity and efficient mesenchymal and amoeboid invasion, respectively [18]. Interestingly, the protein seems to have distinct effects in different tissues and operates both as an oncogene and a tumour suppressor, dependent on its cellular context. Whereas ARA55 in breast cancer promotes invasion and cancer development [16,19], its expression is reduced in colon tumours. In gut epithelial cells, it collaborates with PPARγ to induce expression of markers of gut maturation, consistent with a role as a tumour suppressor [10].

In the present work, we have expanded the list of nuclear partners of ARA55 to include the kinase HIPK1 and the TF c-Myb. Homeodomain-interacting protein kinases (HIPK1-3) constitute a small family of nuclear serine/threonine kinases, initially identified as corepressors for homeodomain TFs of the NK class [20]. HIPK2 is the best-studied member of the family [21]. Judged from its expanding number of phosphorylation targets [22-24], it seems that the HIPK family plays critical roles in transcriptional regulation. It has also been linked to apoptosis, DNA-damage [22] and recently to cytokinesis [25].

The other nuclear partners of ARA55 analysed in this work is c-Myb, belonging to a group of early hematopoietic TFs. It operates as a regulator of stem and progenitor cells in the bone marrow as well as in colonic crypts, and in a neurogenic region of the adult brain [26,27]. It is required for normal adult hematopoiesis and plays a direct role in lineage commitment, cell cycle progression, and differentiation of both myeloid and B and T lymphoid progenitor cells [28,29]. Moreover, c-Myb controls intestinal stem cell genes and self-renewal [30] and is a key regulator that permits early multilineage differentiation of airway epithelial cells [31]. In an oncogenic context, c-Myb enhances the proliferation and blocks differentiation of the cancer cells. Clinical studies have revealed strong links between c-Myb aberrations and human cancer, such as acute myelogenous leukemias, melanomas, and breast, colon and pancreatic carcinomas [26,27,32,33]. Zuber et al. identified c-Myb as a critical mediator of oncogene addiction in acute myeloid leukemia (AML) [34]. Furthermore, c-Myb was found to cause enhanced motility and invasion and to be linked to the EMT response [35-37].

Key coregulators of c-Myb and many other TFs are the acetyltransferases p300 and CBP, acting both as bridges to the basal transcriptional apparatus and facilitating transcription by acetylation of chromatin and its interacting partners [38-42]. Abolishing the interaction between c-Myb and p300/CBP results in non-functional haematopoiesis with thrombocytosis and impaired lymphoid development [43]. A mutation preventing interaction between c-Myb and p300 also prevents transformation and leukemia induction by human AML oncogenes [44]. c-Myb is also reported to bind to the methyl transferase MLL through its complex partner menin, thereby promoting MLL-associated leukemogenesis [45].

In the present work, we first identified ARA55 as a novel interaction partner of the nuclear kinase HIPK1. Together they had a coactivator effect on c-Myb. Interaction assays showed that ARA55 physically associates with c-Myb as does HIPK1. In search of a function for these associations, we detected a novel physical interaction between p300 and ARA55. Together, c-Myb, p300, HIPK1 and ARA55 caused strong synergistic activation of a chromatinized reporter gene, to which they were efficiently recruited. Our data suggest that ARA55 and HIPK1 assist c-Myb in recruiting p300 to chromatin. In support of this hypothesis, we found that c-Myb and ARA55 share a common set of target genes in a cell type where both are expressed.

2. Materials and Methods

2.1. Cell culture, transfection and luciferase assays

Five cell lines were used: CV-1 (ATCC ® CCL-70™ *Cercopithecus aethiops* kidney Normal), COS-1 (ATCC ® CRL-1650™ *Cercopithecus aethiops* kidney), K562 (ATCC® CCL-243™*Homo sapiens* bone marrow, chronic myelogenous leukaemia), HEK-293 (ATCC ® CRL-1573™ *Homo sapiens* embryonic kidney) and U2OS (*Homo sapiens*, epithelial). The latter was a kind gift from Dr. Ian G. Mills, Centre for Molecular Medicine Norway. The cells were grown and transiently transfected with the indicated plasmids as previously described [46,47]. Reporter assays in transiently transfected CV1 and HEK293 cells, the latter stably transfected with a 5×Gal4-luciferase reporter, were performed in triplicate (24-well trays, 2×10^4 CV-

1 cells/well or 3.2×10^4 HEK293-c1 cells/well) using Luciferase Assay Reagent (Promega), each triplicate repeated in three independent experiments.

K562 cells stably expressing V5-tagged ARA55 were generated by standard methods. K562 cells were transfected with linearized pCIneo-ARA55-V5 using the Amaxa kit V (Amaxa, Cologne, Germany) according to manufacture's guidelines. K562 cells stably expressing tagged ARA55-V5 were selected by G418 (400 μg/ml) for 2 weeks before selecting single clones for one week. Stable expression was confirmed by Western blotting.

2.2. Plasmid constructs

The construct for mammalian expression of V5-tagged human ARA55, pCIneo-ARA55-V5, was made by subcloning a PCR-amplified ARA55 cDNA, in which a V5-encoding sequence was added through the reverse PCR primer. The pGEX-6P2-ARA55-V5 was constructed with a similar approach and used to produce recombinant GST-ARA55-V5. Truncated versions (GST-ARA55(1-213) and GST-ARA55(214-461)-V5) were expressed from the plasmids pGEX-6P2-ARA55-N and pGEX-6P2-ARA55-V5-C, which were similarly constructed from PCR amplified ARA55 cDNA.

Plasmids for expression of HIPK1 full-length, wild-type and kinase-dead mutant K219A, has been described [48]. To make the Gal4-fusion bait plasmid for the yeast two-hybrid screening, we subcloned a region of HIPK1 encoding the C-terminal half of the protein (amino acids 632-1209) into pDBT [49] to create pDBT-hHIPK1-BC. Plasmids for expression of p300 full-length and domains have been described [50]. The plasmids encoding Gal4-fusions and GST-fusions of human c-Myb have been described [46,50,51]. The same apply to the reporter plasmids used, pGL4b-3xMRE(GG)-myc [46] and pGL3-GATA2 [52].

2.3. Yeast two-hybrid screening

The two-hybrid screening was performed as previously described [48,53-55]. The interactions were confirmed by transformation of pDBT-hHIPK1-BC and rescued interaction candidates in pACT2 into yeast strains of opposite mating type followed by mating. Diploids were grown on yeast minimal medium supplemented with 0 - 5 mM 3-aminotriazole and lacking the selection amino acids or adenine.

2.4. Antibodies

For immunoblot detection the following antibodies were used: mouse anti-V5 monoclonal antibody (R96025, Invitrogen), mouse anti-FLAG M2 monoclonal antibody (F3165, Sigma-Aldrich), mouse anti-Myb (5E11, [56]), rabbit anti c-Myb H141 (sc7874, Santa Cruz Biotechnology), mouse anti GAPDH (AM4300, Invitrogen), anti-mouse IgG-HRP (715-035-150, Jackson ImmunoResearch), and anti-rabbit IgG-HRP (711-035-152, Jackson Immuno-Research). Protein A Dynabeads (10002D, Invitrogen) and rabbit anti-p300 (C-20, SC-585 X, Santa Cruz Biotechnology) were used for immunoprecipitation. In the ChIP assays, the same rabbit anti-p300 was used in addition to rabbit IgG (EB-003-0110, Diagenode).

2.5. Protein expression and GST pull-down

GST and GST-fusion proteins were expressed in *Escherichia coli* as previously described [57]. GST pull-down assays were performed as described [54] using either the F-buffer [54] or replacing it with a KAc-interaction buffer (150 mM KAc, 20 mM HEPES pH 7,4, 10 % Glycerol, 0,2 % Triton-X-100, 1 mM DTT, 1x

Complete protease inhibitor), using total cell extracts from COS-1 cells transfected with the indicated expression constructs.

2.6. Co-Immunoprecipitation

Co-immunoprecipitations were performed essentially as described [55] using either lysates from transfected COS-1 cells and F-buffer [54] or using the KAc-interaction buffer and lysates from transfected HEK293-c1 cells. Proteins were separated by SDS-PAGE and detected with immunoblotting. Antibodies are as given above.

2.7. Chromatin immunoprecipitation

A HEK293-c1 reporter cell line harbouring a 5×GRE Gal4-luciferase reporter integrated transgene [58] was co-transfected with an HA-tagged Gal-fusion derivative of c-Myb, 3×FLAG-tagged HIPK1, V5-tagged ARA55, and myc-tagged p300. The ChIP analysis was performed as previously described [50]. In short, 24 hours after transfection, cells were crosslinked and nuclei isolated before sonication using a Bioruptor (Diagenode). Samples were incubated with the indicated antibodies overnight, before wash and purification of the isolated DNA using the "ChIP DNA Clean & Concentrator" kit (Zymo Research). Enrichment analysis was performed on a LightCycler® 96 Real-Time PCR System (Roche) using Ultra-Fast SYBR Green QPCR Master Mix (Agilent) and analysed with the supplied software. For primers used, see [46].

2.8. RNA interference

U2OS cells were seeded in 24 well plates one day prior to transfection with 75 µM siRNA against *MYB* and *TGFB1I1* (*ARA55*) (Dharmacon) or siLuc-RNA control using X-tremeGENE siRNA (Roche). After 30 hours cells were harvested and RNA purified and subjected to cDNA conversion using AffinityScript cDNA synthesis kit (Agilent) with random primers.

Quantity real-time PCR analysis was performed with a Lightcycler 96 (Roche) using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent) and analysed with the supplied software. Sequences for primers and siRNAs and additional information are described previously [52,59], and available upon request.

3. Results

3.1. TGFB1I1/ARA55/HIC-5 is a novel interaction partner of HIPK1

In an effort to extend the interaction network of c-Myb, we performed a Y2H screening using one of its interaction partners, HIPK1 [48], as bait. To facilitate isolation of HIPK1-specific interaction partner, we used as bait the C-terminal half of HIPK1, which is the least conserved part of the protein (Fig. 1A). Screening of a human thymus library with Gal4-DBD fused to HIPK1-[676-1209], expressed from a low-copy CEN plasmid, resulted in 320 His-positive clones of which only two grew under stringent conditions in the presence of 5 mM 3-AT (i.e. on -trp-leu-his-ade+5mM 3-AT plates). One of the strongest interacting candidates was ARA55. This is a LIM-domain protein composed of four LD- and four LIM-domains as illustrated (Fig. 1A). The interaction between HIPK1 and ARA55 was first verified in yeast by retransformation and growth on reporter-selective media and by an X-gal overlay assay (data not shown).

In order to validate and map this putative interaction in more detail, we performed a series of interaction assays. First, the association between full-length ARA55 and HIPK1 was validated in a GST-pulldown assay using GST-ARA55 incubated with a lysate from COS-1 cells expressing 3xFLAG-tagged HIPK1 (Fig. 1B). The interaction was also validated using a co-immunoprecitation assay in which we detected V5-tagged ARA55 associated with the immunoprecipitated 3xFLAG-HIPK1 (Fig. 1C). The same was observed in an inverse order co-IP experiment, where 3xFLAG-HIPK1 was detected in the immunoprecipitated V5-tagged ARA55 (Fig. 1C). To further map the interacting part of ARA55, we dissected ARA55 and tested the four LD-domains versus the four LIM-domains of ARA55, both fused to GST. Binding to HIPK1 was only observed with the LIM-domains (Fig. 1B). Hence, these interaction assays support the Y2H screening result and define ARA55 as a novel interaction partner of HIPK1.

3.2. ARA55 interacts with c-Myb

We have previously reported HIPK1 to be an interaction partner of the transcription factor c-Myb [48]. Having now validated the interaction between ARA55 and HIPK1, we asked whether c-Myb might be a common interaction partner of the two. When we tested the c-Myb-ARA55 association in a GST-pulldown assay, we observed that GST-ARA55 fished out c-Myb from a lysate of COS-1 cells transfected with HA-tagged c-Myb, suggesting an interaction (Fig. 2A). To map in more detail the interacting region in c-Myb, reverse GST pulldown assays were carried out using different domains of c-Myb expressed as GST-fusions (Fig. 2B). When COS-1 lysates containing V5-tagged ARA55 was incubated with six different GST-Myb fragments, the ARA55-protein was significantly retained only with c-Myb domains spanning the central transactivating domain (TAD) region (aa 233-325). This is a key functional region of c-Myb to which the p300 KIX domain has been reported to bind [60,61]. This region also contains a SUMO-interaction motifs (SIM), probably binding a not yet identified transcriptional repressor protein [62]. Since mutants were available that interfere with either the p300 KIX interaction [43] or the SIM-binding factor [62], the mutants were analysed in a pull-down assay to see if they interfered with binding of ARA55. However, none of these abolished the ARA55-interaction, suggesting that ARA55 binds to a distinct part of the TAD region (Fig. 2C).

3.3. ARA55 interacts with p300

Having found that ARA55 interacts with the same region of c-Myb that p300 binds to, we asked whether ARA55 might itself also associate with p300, possibly enhancing the p300-c-Myb interaction. First, we tested two domains of p300, the KIX domain (amino acid residues 566-652) and the BP domain (amino acid residues 1039-1285). We observed indeed binding of GST-p300-KIX to ARA55. No binding was observed to the BP-domain of p300 (Fig. 2D). To further validate and extend this interaction studies to full-length proteins, we performed a co-IP experiment and observed again an interaction. The V5-tagged ARA55 was clearly detected in the p300 immunoprecipitate (Fig. 2E). Finally, we used a K562-derived cell line harbouring a transgene expressing ARA55-V5 to show that also endogenous c-Myb interacts with ARA55 (Fig. 2F). Taken together this implicate that the four proteins c-Myb, HIPK1, p300 and ARA55 share several interaction surfaces as summarized in Figure 2G.

3.4. Functional consequences of ARA55 recruitment to c-Myb

Since ARA55 appears to associate with both c-Myb and its two interaction partners, HIPK1 and p300, we investigated the effect of ARA55 on the transcriptional activity of c-Myb. In a simple luciferase reporter assay in CV1 cells, ARA55 operates as a coactivator of c-Myb, as might be expected from its combined p300 and c-Myb interaction (Fig. 3A). The effect of HIPK1 on c-Myb activity has been difficult to assess since we have observed both activation and repression in different assays (data not shown). For this reason, we decided to perform a fine-tuned gradual increase in the HIPK1 input. As shown in Fig. 3B, we did consistently observe a dual effect by this approach. While the smallest input had no effect, when the HIPK1 level reached a certain threshold, we observed a reproducible drop in both activity and level of c-Myb, hence a repressive effect. When HIPK1 increased further, activation took over and c-Myb gradually became more active at higher inputs of HIPK1. When ARA55 was added in a constant amount to the same assay, this dual behaviour was abolished and we observed a gradual increase in activity of c-Myb and no degradation phenomena (Fig. 3C). It thus appears that the association of ARA55 with c-Myb and HIPK1 abolished the negative effect of HIPK1 and stabilized its coactivator behaviour. When we included the acetyltransferase p300 and tested reporter activation in different combinations, we observed that all three partners, p300, HIPK1 and ARA55, had coactivator effects and the highest activation was observed when all three were added together, both on a synthetic reporter (Fig. 4A) and with a reporter construct containing the promoter for the c-Myb target gene *GATA2* [52] (Fig. 4B).

To analyse further this coactivation, we turned to a chromatinized reporter and used the HEK293-c1 system developed by Suske and co-workers [58], in which an array of Gal4-responsive elements (5xGRE) in front of a luciferase reporter is integrated into the genome of HEK293 cells. To target c-Myb to this site, we used a Gal4-c-Myb fusion. In this system, we observed a dramatically higher fold-activation. The combination of c-Myb and all three partners caused a 150-fold activation relative to c-Myb only (Fig. 4C). By using a "minus one" approach, we concluded that this superactivation was fully dependent on c-Myb, highly dependent on HIPK1, and to a significant degree also dependent on the kinase activity of HIPK1. When ARA55 was taken out, the activity dropped to 50%. Thus it appears that both protein-protein interactions and kinase activity plays a role in

this chromatinized reporter assay. Inspection of the associated western blot revealed a differential stabilization depending on the combination of partners present. The inherently unstable c-Myb protein [65] appears to be stabilized in the presence of p300 and HIPK1, independent of ARA55 (lanes 3, 4 and 6). ARA55 itself shows higher steady state levels when p300 and HIPK1 are present (lanes 3 and 4) than when HIPK1, p300 or c-Myb are left out (lanes 5, 7, and 8). HIPK1 also shows a reduced steady state level when p300 or c-Myb are left out. Altogether this point to a complex interplay between HIPK1, ARA55 and p300 and c-Myb bound to chromatin.

3.5. ARA55 and HIPK1 cause more efficient c-Myb dependent recruitment of p300 to chromatin

Given the network of interactions uncovered, one obvious hypothesis is that the c-Myb interaction partners cooperate to facilitate p300 recruitment and thereby causing enhanced gene activation. Therefore, we directly explored the efficiency of recruitment of p300 to chromatin-bound c-Myb and in particular how this recruitment is affected by the presence of HIPK1 and ARA55. To this end, we performed ChIP in the HEK293-c1 cell line [58] and monitored the occupancy of the three partners both at the c-Myb bound site (5xGRE) and at a more distant locus (*NCOA5* intron) serving as background reference. When tested pairwise (partner+c-Myb), the assay showed only a modest recruitment of ARA55, HIPK1 and p300 to the chromatin site where c-Myb was bound. However, in presence of both ARA55 and HIPK1 the recruitment of the co-regulator p300 was increased fourfold (Fig. 5D). Concomitantly, the recruitment of HIPK1 and ARA55 was also strongly enhanced. These observations are in line with the interaction experiments (Fig. 1-3) and consistent with the hypothesis that HIPK1 and ARA55 work as mutually stabilising complex partners promoting the recruitment of p300 to chromatin bound c-Myb.

3.6. Knockdown of ARA55 impairs c-Myb dependent gene activation in hematopoietic cells

ARA55 and c-Myb are both cell type specific factors. Targets of ARA55 have been identified genome-wide in U2OS osteosarcoma cells [59], a cell type where also c-Myb is expressed [66], although at a lower level than in hematopoietic progenitor cells [67]. To further explore the biological relevance of the novel interaction between ARA55 and c-Myb, we investigated putative common target genes of the two factors. More specifically, we analysed the expression of six c-Myb target genes (*KCNH2*, *GATA2*, *STAT5A*, *MYC*, *FGFRL1* and *BHLHE40*) [47,52] upon knockdown of the two factors in the osteosarcoma cell line. The analysis showed that four of the six genes were down-regulated upon suppression of either c-Myb or ARA55 (*KCNH2*, *GATA2*, *STAT5A* and *FGFRL1*), while two genes did not show any altered expression (*MYC* and *BHLHE40*) in this cell line (Fig. 6). This shows that c-Myb and ARA55 share some common target genes and therefore should be able to act in concert to control gene programs in cell types where they both are expressed.

4. Discussion

The ARA55 protein has been reported to operate as a dual regulator with functions both in the cytoplasmic and nuclear compartments [1,2]. In this work, we

have expanded its nuclear functions by showing that it interacts with both the nuclear kinase HIPK1, the transcription factor c-Myb and the coactivator and acetyltransferase p300. The interactions of ARA55 with these three regulators were confirmed by both GST pull-down and by CoIP of full-length proteins. In addition, ChIP experiments demonstrated that the interactions operate when bound to chromatin and play a role in a concerted recruitment of p300 to c-Myb-bound sites. The novel interaction of ARA55 with p300 was mapped to the KIX domain of the acetyltransferase, while the interaction to c-Myb was mapped to the transactivation domain of c-Myb, which in fact is the same domain used by the activator for interaction with p300 [60].

The recruitment of p300 appears to be a critical event in the function of c-Myb [43,44,68-70]. However, the affinity of the c-Myb TAD region for the KIX-domain in p300 is significantly weaker than what is observed for the phosphorylation-dependent interaction between KIX and the transcription factor CREB, being a critical event in triggering c-AMP dependent gene programs [60,71-74]. This creates a regulatory window in which other associated factors may strengthen the c-Myb p300 interaction. One example is the dual binding of p300 and the mixed lineage leukemia (MLL) protein creating an active ternary MLL:KIX:c-Myb complex [72,75,76]. We have recently shown that PIAS1 may exert such a stabilizing function [50] and have in the present work concluded that ARA55, together with HIPK1, exerts an analogous stabilizing effect. In this way, ARA55 contributes to the transactivation function of c-Myb by enhancing its ability to recruit a key coactivator. ARA55 have been studied in relation to p300 or CBP also in the context of nuclear receptors, but with somewhat deviating results. Direct physical interaction has not been reported previously. In line with the situation for c-Myb, Heitzer et al. observed reduced recruitment of p300 to a genomic locus bound by the glucocorticoid receptor upon depletion of ARA55 suggesting a contribution to the recruitment of the coactivator [13]. In contrast, Chodankara et al. found that ARA55 knockdown did not influence the occupancy of p300 but instead affected the recruitment of the basal transcription factor machinery to the locus [59]. Moreover, ARA55 was found to enhance acetylation the nuclear receptor TR4 in its DNA-binding domain, via recruiting proteins with HAT activity, in this case leading to suppression of TR4 transactivation [77]. The role of ARA55 in transcriptional control may well be gene and context specific as often is the case for this class of regulators.

ARA55 and c-Myb are both cell type specific factors with restricted expression profiles. A critical question is therefore whether they operate in the same cell type. The biological functions of c-Myb have traditionally been studied mainly in the hematopoietic system [27,78,79], where ARA55 is not highly expressed. This may therefore not be the most relevant tissue for the cooperation between the two factors. However, c-Myb has been found to operate in a much broader range of cell types, such as epithelial cells, vascular smooth muscle cells, prostate and breast cancer cells and progenitor cells in colonic crypts [26,31,33,80-83]. ARA55 is well expressed in various epithelial cells and cells of muscle, prostate, breast and colon [84] (http://fantom.gsc.riken.jp/5/sstar/EntrezGene:7041). This situation may resemble the interplay of ARA55 and the androgen receptor. Here the role of ARA55 is complex, possibly functioning as a stromal-specific AR coactivator with a role in regulating expression of the growth factor and/or cytokine expression in the prostate [3] and where epithelial ARA55 expression contributes to prostate

tumorigenesis and castrate responsiveness [12]. In the present work, we have used the osteosarcoma cell line U2OS where both c-Myb and ARA55 are expressed [59,66]. We found that a selection of previously reported c-Myb targets did respond very similarly to knockdown of c-Myb and ARA55, strongly suggesting that the two interacting regulators share common target genes in a physiological context. This argues for the biological relevance of the mechanisms analysed in this work.

Further studies are needed to dissect the biological processes being under the dual control of both c-Myb and ARA55. One interesting candidate is the epithelial-to-mesenchymal transition (EMT) process, during which epithelial cells acquire characteristics of mesenchymal cells, a process that is being implicated both in normal development and in tumour progression and metastasis [85,86]. It is well established that ARA55 plays a role in EMT as summarized in the Introduction. Recently, c-Myb has also been implicated in the same process through its regulation of EMT-associated genes and genes linked to invasion and metastasis [35,36,87-90]. Future studies will dissect how these processes are linked in more mechanistic detail.

The precise role of HIPK1 in the gene activation has not been studied in depth in the present work beyond showing it being involved in the p300 recruitment process. It may be of notice that the kinase, like ARA55, may operate both in the cytoplasm and the nucleus in a signal-dependent manner. TNF triggers its translocation to the cytoplasm in response to stress stimuli, promoting apoptosis [91]. Of notice is also that we, in the same Y2H screen using HIPK1 as bait, isolated another LIM-domain protein, Zyxin, but did not study this partner further since it is already reported to be an interaction partner of HIPK2 with a role in the apoptotic HIPK2-p53 signalling axis [92]. Interestingly, zyxin is also a dynamic component of integrin-based focal adhesions and as well as of stress fibers [93]. Recently, it has been hypothesized that LIM proteins are mechanoresponders [93]. How this complex network of signal-modulated proteins operates in a concerted action will need extensive further studies in relevant models.

5. Conclusions

We report that ARA55 has more nuclear interaction partners than previously known. We conclude that c-Myb, ARA55, p300 and HIPK1 interact with each other through specific domains. On chromatin, these interactions caused more efficient recruitment of p300 to c-Myb-bound loci. ARA55 and c-Myb share a common set of target genes in osteosarcoma cells. Hence, in addition to its function in the cytoplasmic compartment, ARA55 clearly operates as a complex transcriptional regulator in the nucleus, being able to fine-tune the transcriptional output of other factors in a highly context-dependent manner.

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7. Legends to Figures

Figure 1. Human ARA55 interacts with HIPK1 through the LIM domains of ARA55. (**A**) HIPK1 and ARA55 are depicted with their domain structures. S: SUMO, SIM: SUMO-interaction motif. (**B**) GST pull-down binding assays were performed with different GST-ARA55 domains as indicated and FLAG-tagged human HIPK1 from transfected COS-1 cells. The bound proteins were separated by SDS-PAGE and the immunoblot was analysed using anti-FLAG antibody (1:10,000) and secondary anti-mouse-HRP antibody (1:10,000). 3 % of total cell extract used for each pull-down was loaded as the reference. The loadings of the GST-proteins were controlled by staining the blot with Ponceau S (right panels). (**C**) Coimmunoprecipitation experiments were performed with COS-1 cells. The cells were transfected with plasmids encoding 3xFLAG-HIPK1 and ARA55-V5 and lysed 24 hour after transfection. Immuno-precipitated proteins were separated by SDS-PAGE and detected on immunoblot using anti-FLAG antibody (1:10,000) or anti-V5 antibody (1:5000) and secondary anti-mouse-HRP antibody (1:10,000). 1 % of total transfected cell lysate was loaded as input reference.

Figure 2. ARA55 interacts with c-Myb through its TAD domain and with p300 through its KIX-domain. (A-D) The GST pull-down experiments were performed as described in the legend to Figure 1, with lysates from COS-1 cells transfected with V5-tagged human ARA55 or with HA-tagged c-Myb and immobilized GST-fusion proteins as indicated (hcM: human c-Myb). The loadings of the GST-proteins were controlled by staining the blots with Ponceau S or Coomassie Brilliant Blue. (E) Coimmunoprecipitation experiments were performed with COS-1 cells transfected with plasmids encoding pCMVβ-NHA-p300 and ARA55-V5 and lysed 24 hour after transfection. Proteins were immunoprecipitated with anti-p300 antibody, separated by SDS-PAGE and detected on immunoblot using anti-V5 antibody (1:5000) and secondary anti-mouse-HRP antibody (1:10,000). 5 % of total transfected cell lysate was loaded as input reference. (F) Semi-endogenous coimmunoprecipitation experiments were performed with K562 cells, parental or the A6 clone with an integrated transgene encoding ARA55-V5. The indicated combinations of immunoprecipitation (IP) and immunoblotting (IB) are shown. (G) Schematic representation of the contact points between p300, ARA55, HIPK1 and c-Myb. Interactions are indicated by arrows, some of which were identified in the present work (marked "1."), some are based on previous reports (2. = [60], 3.= [48] and 4.= [63,64]).

Figure 3. ARA55 and HIPK1 cooperate to enhance c-Myb transcriptional activity. (A) CV-1 cells were transfected with the synthetic c-Myb responsive 3xMRE-(GG)-Myc reporter plasmid and plasmids encoding c-Myb (0.2 µg) and ARA55 (0.1-0.4 µg), as indicated. Western immunoblotting (IB) is shown in the lower panel. (B) CV-1 cells were transfected with the same reporter construct as in (A), a fixed amount of a c-Myb encoding plasmid (0.2 µg) and increasing inputs of a plasmid encoding HIPK1 as indicated. Western immunoblotting is shown in the lower panels. (C) The same setup as in (B) but now with a fixed additional input (0.2 µg) of a plasmid encoding ARA55. All luciferase results are presented as relative luciferase units (RLU) ± SD of at least three independent assays performed in triplicate.

Figure 4. ARA55, HIPK1 and p300 cooperate to enhance c-Myb transcriptional activity. (A, B) CV-1 cells were transfected with combinations of plasmids encoding c-Myb, ARA55-V5, 3xFLAG-HIPK1 or p300 as indicated. As luciferase reporter was used either a construct driven by (A) a synthetic c-Myb responsive 3xMRE(GG)-*Myc* promoter or (B) one driven by the c-Myb responsive *GATA2* promoter. Western blot analysis of the combinations of transfections in CV-1 cells used in panel is shown. (C) HEK293-c1 cells with a 5xGRE-luciferase gene integrated into the genome were transfected with plasmids encoding Gal-c-Myb (194-640) (marked "c-Myb"), 3xFLAG-HIPK1 wild-type or kinase dead mutant (K219A), ARA55-V5 (all 0.1 μg) and p300-myc (0.2 μg) in the combinations indicated. Western blot analysis of the combinations of transfections HEK293-c1 cells is shown in the lower panel. All luciferase results are presented as relative luciferase units (RLU) ± SEM of at least three independent assays performed in triplicate. Significance was evaluated by unpaired, two-tailed t-tests on selected pairs and indicated with p-values (*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$; ns $p > 0.05$).

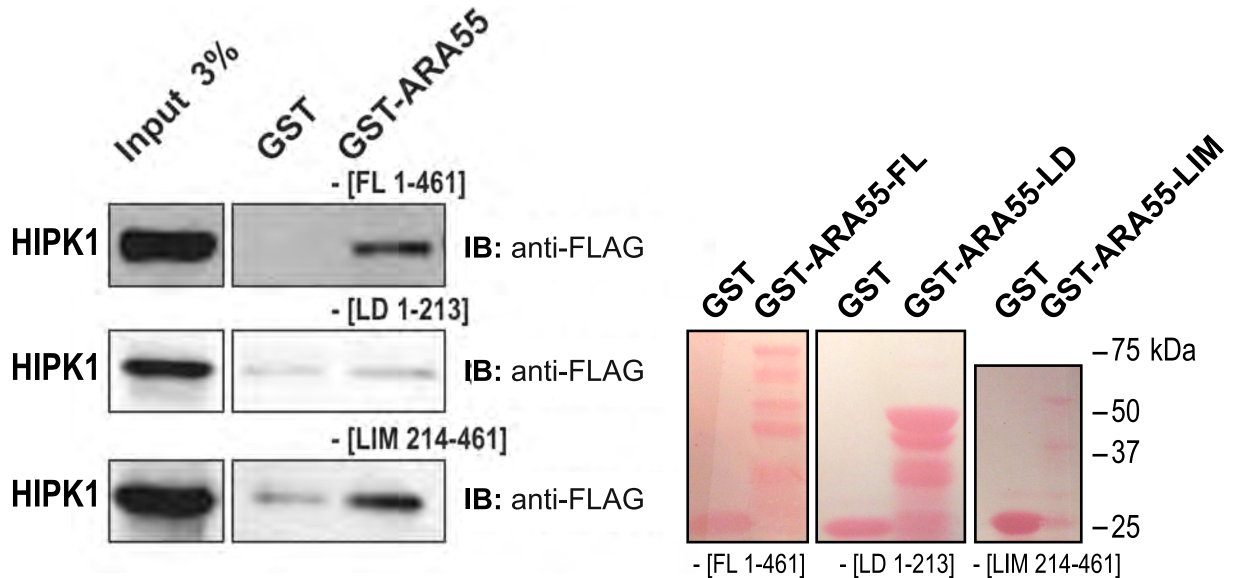
Figure 5. ARA55 enhances recruitment of p300 and HIPK1 to c-Myb bound chromatin. ChIP assay performed in HEK293-c1 cells with an integrated 5×GRE-Luciferase gene. (A) Illustration showing the genomic loci in the HEK293-c1 cells with the integrated 5×GRE-Luciferase gene and the downstream *NCOA5* intron used as control (drawing based on Fig.2 in [58]). (B-D) ChIP assay of ARA55, HIPK1 and p300 recruitment to chromatin. HEK293-c1 cells were transfected with Gal-c-Myb (1-640), p300, ARA55-V5 and 3×FLAG-HIPK1 in the indicated combinations. 24 hours after transfection cells were crosslinked and processed for ChIP analysis as described in Material and Methods. Immunoprecipitations were performed against each of the three factors: B) ARA55, C) HIPK1 and D) p300 using the antibodies listed in Material and Methods. Occupancies of each factor on the 5×GRE promoter and on the *NCOA5* intron were analysed using ChIP-qPCR. The results are shown as the mean of two independent biological assays with ± SEM. E) Western blot analysis of the combinations of transfections used in the HEK293-c1 cells.

Figure 6. c-Myb and ARA55 share a common set of target genes in the osteosarcoma cell line U2OS. (A) U2OS cells were transfected with control siRNA or siRNA targeting MYB and ARA55 respectively. The mRNA levels of the two genes were measured by quantitative real-time PCR using primers specific for the targeted genes and the reference gene TBP. (B) The mRNA levels of the six c-Myb targets genes KCNH2, FGFRL1, GATA2, STAT5A, BHLHE40 and MYC were measured similarly in U2OS cells treated with control siRNA or siRNA against MYB and ARA55, respectively. Expression levels were normalized against the reference genes. The data shown is the average of two biological replicates ± SEM. Significance was evaluated by unpaired, two-tailed t-tests on selected pairs and indicated with p-values as in Figure 4.

Figure 1

A.

B.



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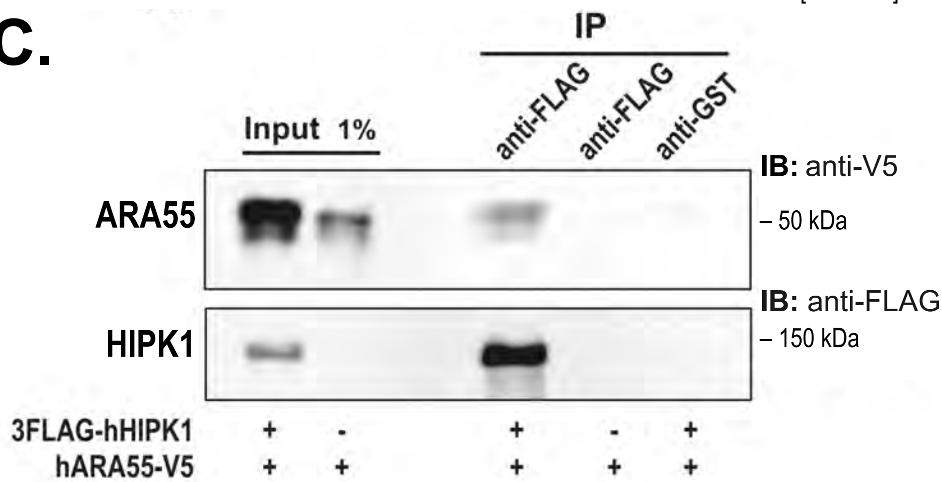


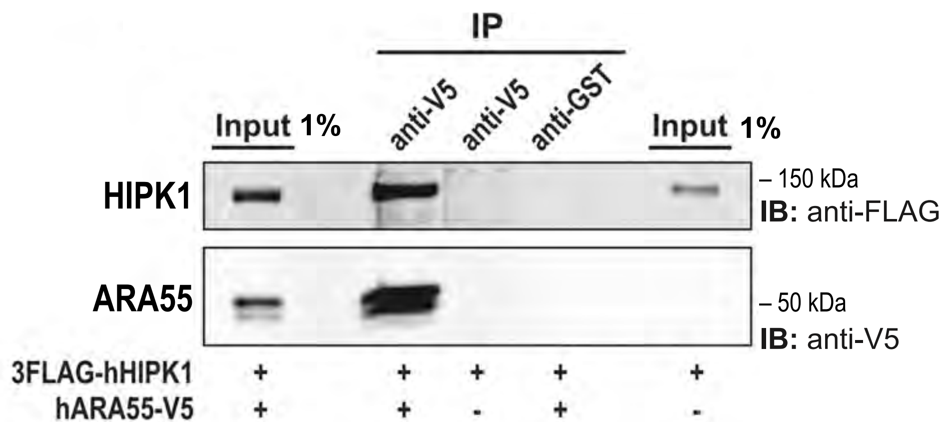


Figure 2

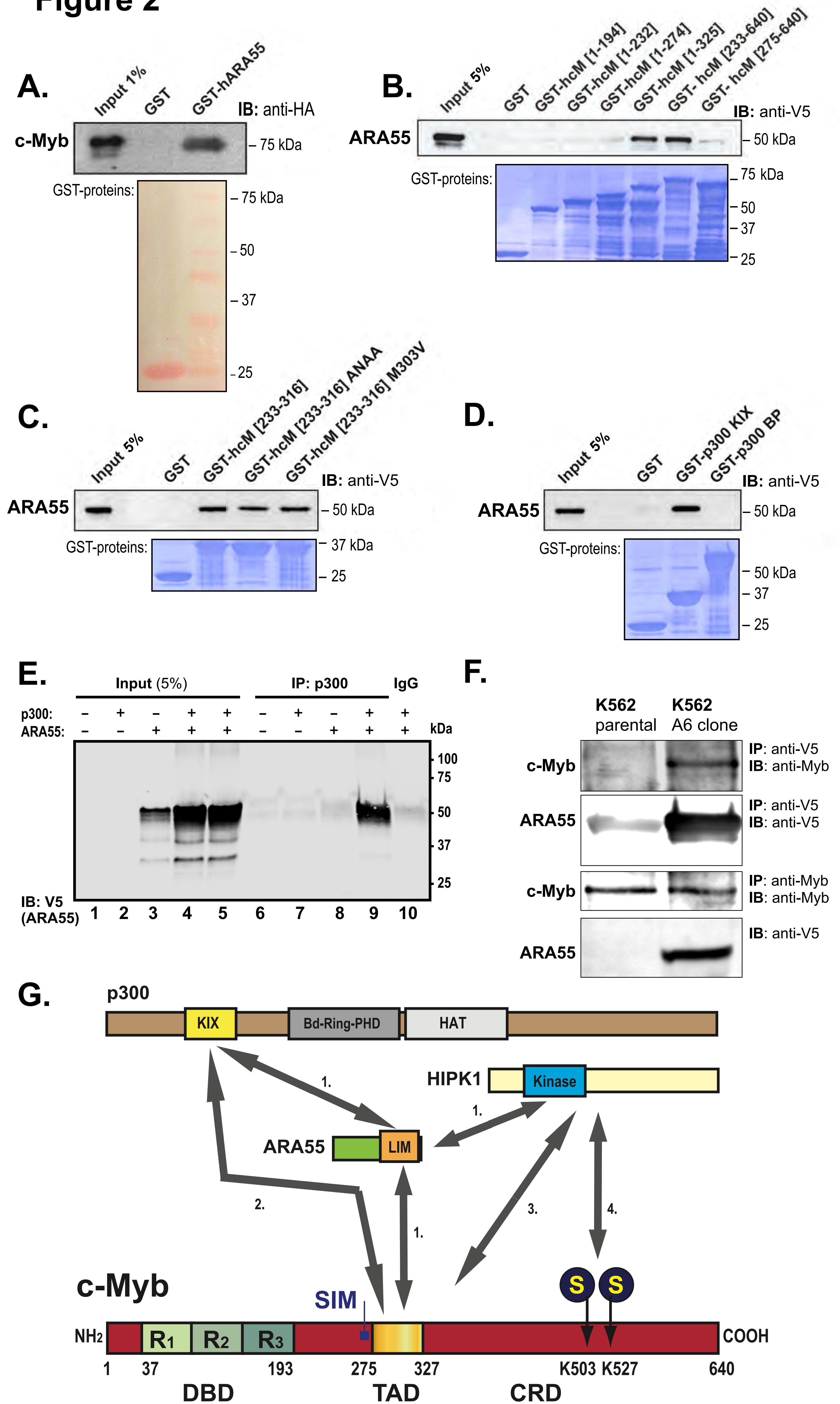
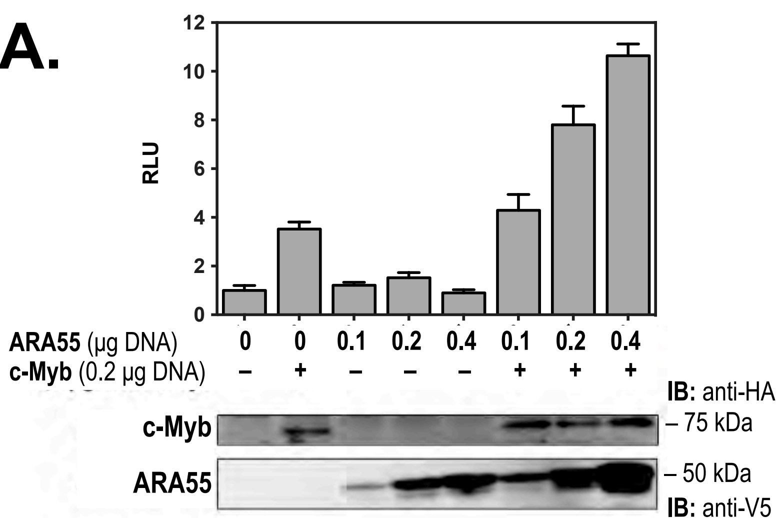
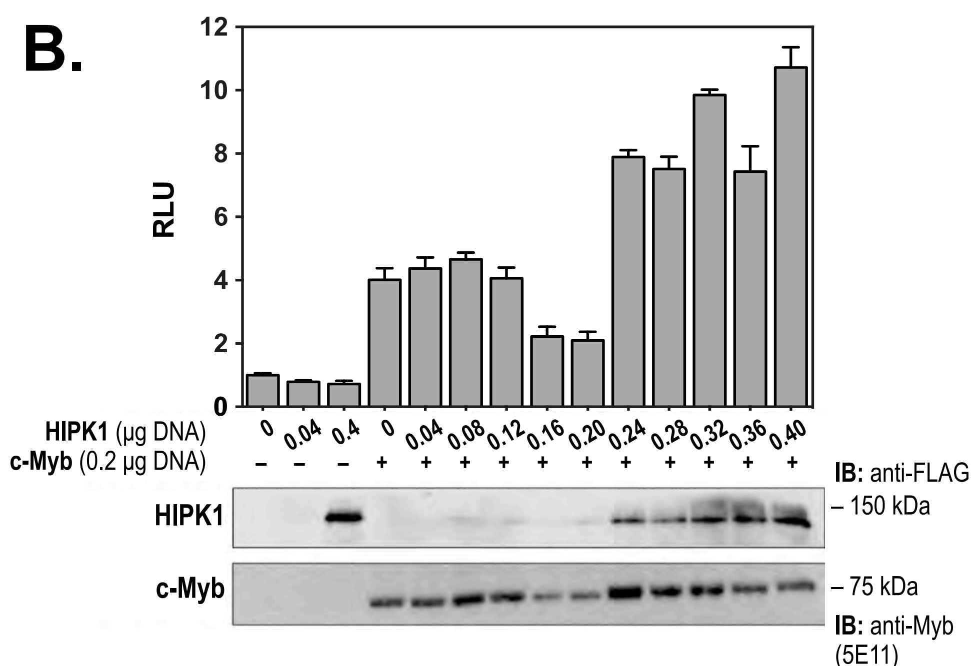


Figure 3





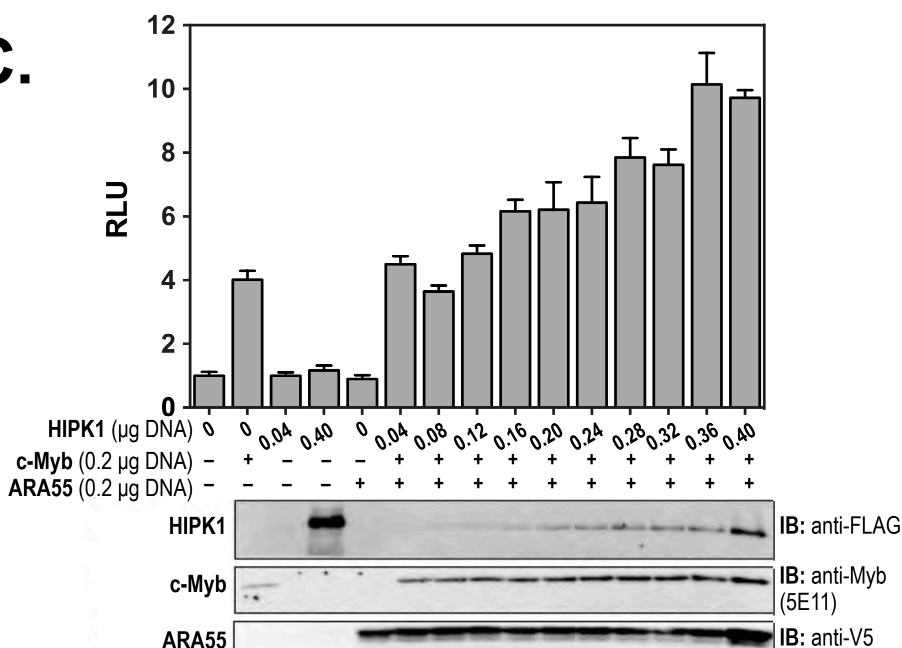


Figure 4

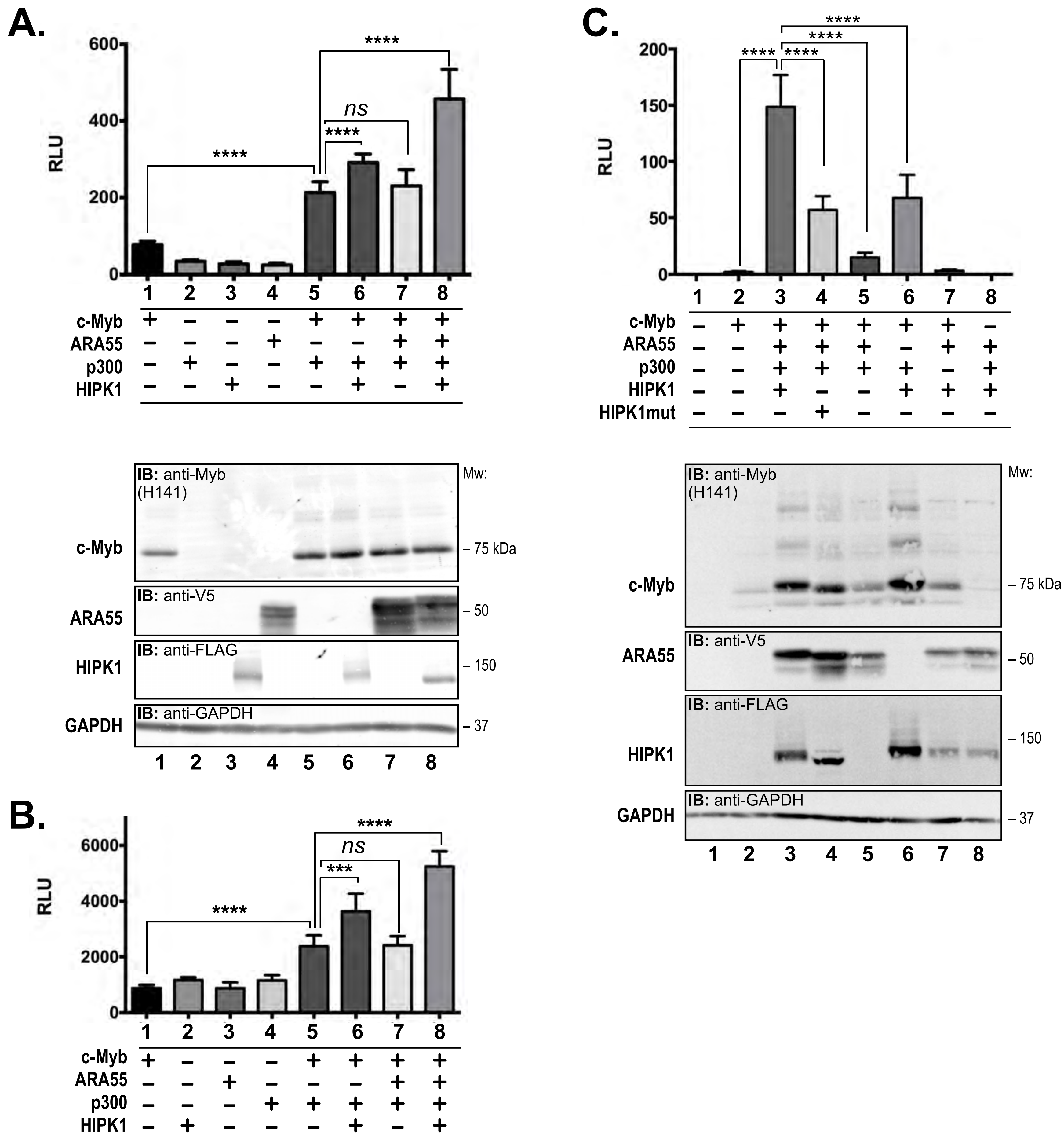
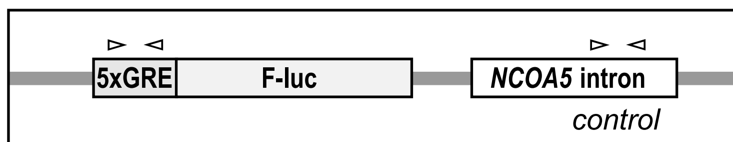
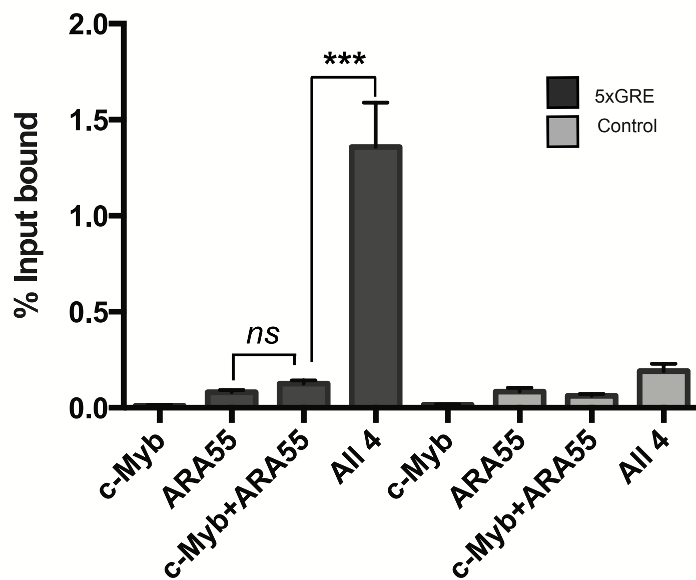
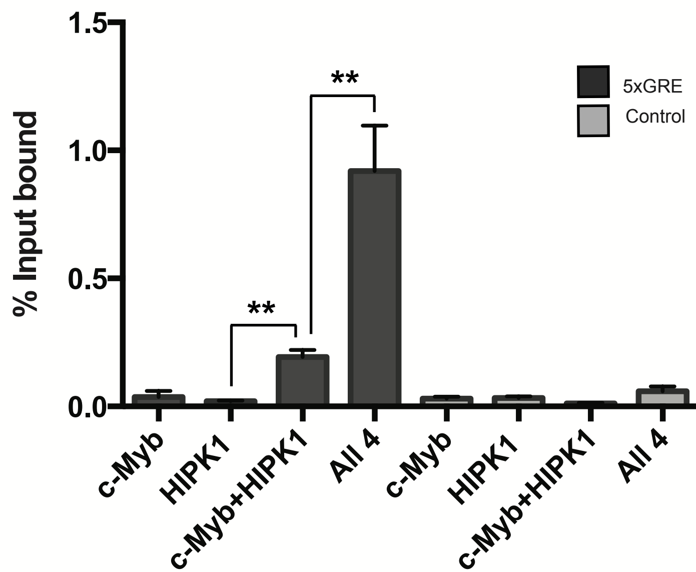
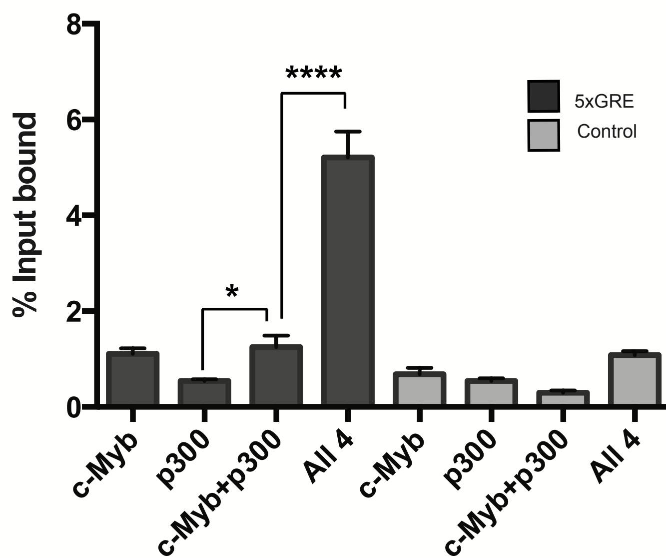


Figure 5









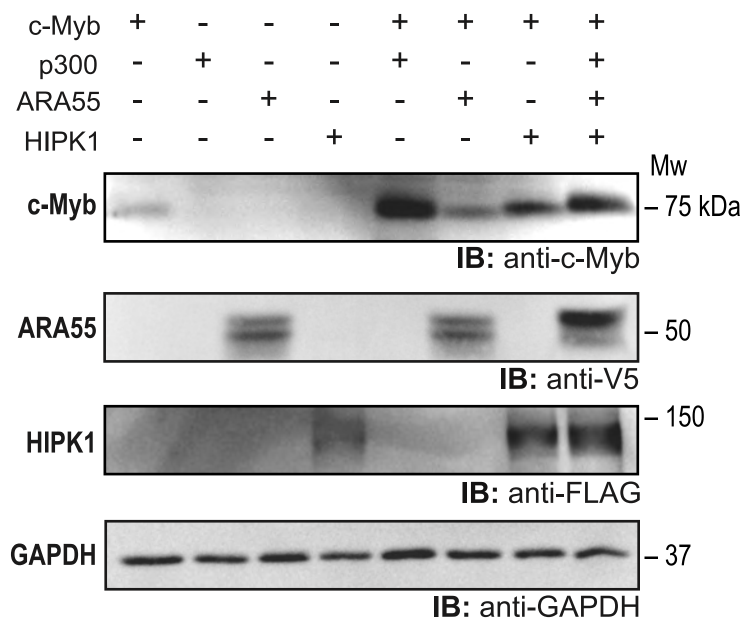


Figure 6