Low-density lipoprotein receptor-related protein interacts with MafB, a regulator of hindbrain development

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Abstract The intracellular domain (ICD) of the low-density lipoprotein receptor-related protein (LRP) functionally interacts with adaptor proteins both as an integral part of the receptor polypeptide and after proteolytic release. Identification of such adaptors has been difficult because the ICD is self-activating in conventional transcription factor-based yeast two-hybrid screens. We adopted an alternative screen for the ICD that depends on the activation of the Ras-signaling pathway and uncovered the transcription factor MafB as novel ICD interacting protein. MafB is a regulator of hindbrain segmentation and interacts with the ICD through a leucine zipper domain. The ICD co-localizes with MafB to the nucleus and negatively regulates its transcriptional activity, suggesting a possible role for LRP in brain development.

Key words: Leucine zipper domain; Intracellular domain; Kreisler; Low-density lipoprotein receptor gene family; Yeast two-hybrid screen; Transcription factor

1. Introduction

The low-density lipoprotein (LDL) receptor-related protein (LRP) is a 600 kDa cell surface receptor structurally related to the LDL receptor [1]. It constitutes a multifunctional receptor pathway that binds many ligands and affects various activities in tissues that express the protein [2,3]. In hepatocytes, LRP acts as a clearance receptor for chylomicron remnants, lipoproteins carrying dietary lipids [4]. In vascular smooth muscle cells, it regulates the activity of the platelet-derived growth factor receptor-β and affects atherosclerotic lesion formation [5–7], while in neurons it modulates processing of the amyloid precursor protein (APP) and may play a role in Alzheimer's disease [8,9]. The embryonic lethality in mice with LRP gene defect suggests additional functions during development that still await further elucidation [10].

Recently, much attention has been focused on the intracellular domain (ICD) of LRP as an important determinant of receptor activity. This domain consists of 100 amino acids that form a number of recognition motifs for interacting proteins. Among others, it includes two NP*X*Y elements that are subject to tyrosine phosphorylation and constitute binding sites for proteins with phosphotyrosine binding (PTB) domain [5,7,11,12]. Through binding of adaptor proteins, the ICD forms multimeric protein complexes at the plasma membrane that are considered crucial for the various receptor activities [2,3]. In addition, this domain can be cleaved from the receptor and act as a modulator of transcription by yet unknown mechanisms [13,14].

Given the central role of protein–protein interactions at the ICD, identification of binding partners for the receptor tail remains an important task in elucidating the molecular details of receptor function. This task has been confounded by the fact that the LRPICD is self-activating in conventional yeast two-hybrid screens that rely on the assembly of an active transcription factor through interaction of the receptor tail (bait) with target proteins (prey) [12]. Therefore, this technology, though widely used to identify novel interaction partners, is not applicable to LRP. Alternative strategies to uncover ICD interacting proteins included the co-purification of the receptor with adaptor proteins such as the PTB-domain protein Shc [11], or the test of candidate adaptors that bound to related receptors using pull-down experiments [12,15].

Because previous candidate-based approaches for interacting proteins may have been biased by our limited knowledge of the receptor biology, we sought to apply an alternative system that would allow an unbiased screen for novel and unexpected interaction partners for the LRPICD. We successfully used a yeast two-hybrid screen that is based on functional interactions in the Ras-signaling pathway and depends on the assembly of protein complexes at the plasma membrane rather than the nucleus. In this screen, we identified the transcription factor MafB as LRP interacting protein. MafB is a regulator of hindbrain development and interacts with the LRPICD through a basic region linked to a leucine zipper domain (bZIP). LRPICD co-localizes with MafB to the nucleus and negatively regulates its transcriptional activity.

2. Materials and methods

2.1. Materials

An expression construct encoding the PTB domain of mammalian disabled 1 (Dab1), fused with glutathione-*S* transferase (GST), was

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provided by Michael Gotthardt (Washington State University). The full-length murine MafB cDNA was produced by RT-PCR from mouse liver RNA.

2.2. Yeast two-hybrid screen

Starting from a mouse LRP cDNA sequence [16], a LRPICD encoding fragment (amino acids 4446–4545) was generated by a PCR approach, cloned into vector pSos (LRPICD/pSos, Stratagene, www.stratagene.com) and used as a bait to screen a human fetal brain library (No. 975230) according to the manufacturer's recommendations (CytoTrap™, Stratagene). Positive clones were isolated from the yeast strain cdc25H and their interaction with the LRPICD confirmed by retransformation of the plasmids in the presence of either LRPICD/pSos or pSos. Truncations of clone 17 were generated by PCR cloning approach, introduced into target vector pMyr, and their interactions with LRPICD determined by transformation of the respective constructs into yeast strain cdc25H.

2.3. Expression of recombinant proteins

GST and GST-fusion proteins of clone 17 or murine MafB were obtained by cloning of the respective gene sequences into vector pGEX-4T-1 (Amersham, www.amershambiosciences.com) and expression in DH5α bacteria. Recombinant proteins were purified by glutathione–agarose affinity chromatography. A fusion of the LRPICD with a hexa-histidine epitope (His-LRPICD) was produced by cloning of the ICD encoding sequence into vector pET16b (Novagen, www.novagen.com) and by purification of the fusion protein from BL21 bacteria via routine Ni–NTA affinity chromatography.

2.4. In vitro protein interaction

Ligand blot analysis was performed as described [17]. Iodination of His-LRPICD followed the protocol of Fraker and Speck [18]. For surface plasmon resonance analysis, purified His-LRPICD was immobilized on the CM5 sensor chip surface and incubated with the indicated concentrations of GST-clone 17 or GST-Dab1 as published [17].

2.5. Immunocytochemistry

The MafB encoding cDNA sequence was introduced into vector pEGFP-C1 (BD Biosciences, www.bdbiosciences.com) and expressed as a fusion with the enhanced green fluorescent protein (EGFP). Sequences encoding LRPICD were cloned into pDsRed2-C1 (BD Biosciences) and expressed as fusion with the Discosoma red fluorescent protein (DsRed2). For expression studies, HEK 293 cells were transfected with 0.5 μg/12-well of the plasmids by liposomal transfection technology (Fugene 6; Roche, www.roche.com). Expression and subcellular localization of the proteins were confirmed by confocal fluorescence microscopy (Leica TCS SP2).

2.6. Reporter gene assay

The reporter gene plasmid was produced by introducing an oligonucleotide sequence containing three consecutive repeats of a Maf recognition element (MARE) into the pTALSEAP vector (BD Biosciences) driving expression of the secreted human placental alkaline phosphatase (SEAP) gene (MARE/pTALSEAP). Following transfection of HEK 293 cells (48 h), cell pellets and conditioned medium were harvested for reporter gene assays. Conditioned medium was processed according to the manufacturer's instructions and SEAP activity assayed using the EscAPe™ SEAP system (BD Biosciences). For control of transfection efficiency, transfection mixtures included pCMV-β-gal that encodes the β-galactosidase gene under control of the Cytomegalovirus promoter element (Stratagene). After removal of the conditioned medium, cells were washed once with PBS, lysed in 100 μl of reporter lysis buffer (Promega, www.promega. com) per 12-well, and processed according to manufacturer's instructions (Luminiescent β-gal, BD Biosciences).

3. Results

To circumvent the problem of self-activation of the LRPICD in conventional yeast two-hybrid screen (e.g., lexA system), we explored the use of the CytoTrap™ system to screen for LRP interacting proteins. To do so, we expressed the LRPICD as a fusion protein with the human *son of sevenless* (hSos) gene product (LRPICD/pSos) and screened a human fetal brain library where target proteins are tethered to the plasma membrane via a myristylation sequence (pMyr). Functional interaction of LRPICD with target proteins would locate the LRPICD/hSos fusion protein to the plasma membrane, reconstituting the Ras-signaling pathway by functionally replacing the yeast Sos homologue that is defected in the yeast strain cdc25H.

No functional reconstitution of the Ras-pathway was observed when cdc25H cells were transfected with LRPICD/pSos alone, but five positive clones were obtained when co-transfected with the cDNA library in vector pMyr (data not shown). Three clones encoded the EB1 protein, an interaction partner of the APC tumor suppressor protein [19]. The other two clones contained a partial cDNA sequence, encoding the truncated MafB protein lacking the first 147 residues of the 323 amino acid polypeptide (provisionally designated clone 17) (Fig. 1). MafB belongs to the Maf protein family of transcription factors that are characterized by the presence of a bZIP [20,21]. The basic region binds to MARE in the DNA, whereas the leucine zipper domain enables interaction with other proteins. In addition, full-length MafB encompasses an activation domain and two histidine repeats (Fig. 1). Mutations in the murine MafB gene are responsible for the mouse mutant *Kreisler*, a developmental defect of the hindbrain [20].

Because the LRPICD is known to undergo proteolytic processing and translocation into the nucleus, we focused further attention on a possible interaction of the LRPICD with the transcription factor MafB. First, we confirmed the interaction of MafB with the receptor employing ligand blot and surface plasmon resonance (BIAcore) analysis. To do so, a fusion protein of GST and clone 17 (GST-clone 17) was expressed and purified from bacterial extracts by glutathione–agarose affinity chromatography (Fig. 2, lane 3). As a positive control, we expressed a fusion of GST with the PTB domain of Dab1, an established adaptor of LRP [12] (Fig. 2, lane 2). By ligand blot analysis, we demonstrated binding of soluble LRPICD to GST-clone 17 and GST-Dab1 but not to GST (Fig. 2, lanes 4–6). This interaction could be confirmed by surface plasmon resonance analysis. Using a concentration series, a K_d of 1 μM was determined for binding of the soluble GST-clone 17 to the LRPICD immobilized on the biosensor chip surface (Fig. 3A). The same affinity was obtained when using the full-length LRP purified from rat liver (data not shown). As a control, a K_d of 0.3 μM was determined for binding of GST-Dab1 to the LRPICD (Fig. 3B). GST alone did not interact with the receptor (not shown).

To test whether LRPICD and MafB co-localized in cells, we generated fusion proteins of full-length murine MafB with the EGFP and LRPICD with DsRed2 fluorescent protein and expressed them in HEK 293 cells. Using confocal fluorescence microscopy, EGFP-MafB was detected exclusively in the nucleus of the cells, whereas DsRed2-LRPICD was seen both in the cytoplasm and in the nucleus, where it co-localized with EGFP-MafB (Fig. 4).

To determine whether a functional interaction exists between LRP and MafB in the nucleus, we tested the effect of LRPICD on MafB activity using reporter gene assays. We generated a reporter construct containing three copies of a MARE sequence that determines recognition by Maf proteins (TGCTTACTAAGCA) [22], followed by a TATA-like pro-

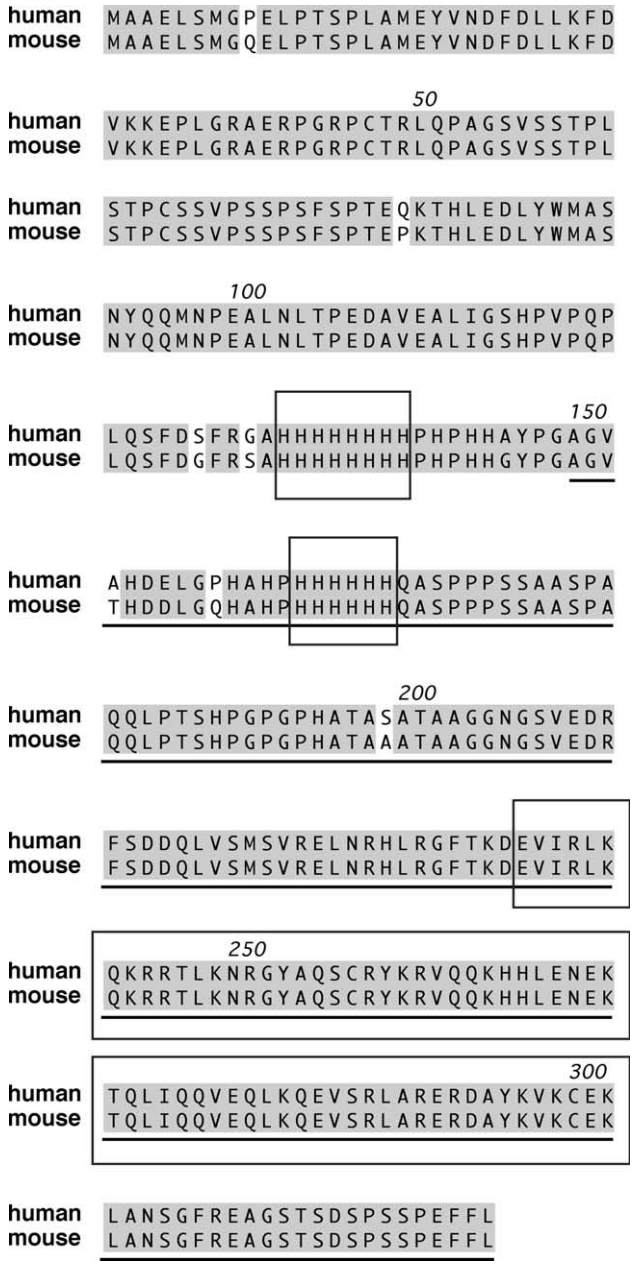


Fig. 1. Sequence and structure analysis of MafB. The amino acid sequences of the human and mouse MafB proteins are depicted. Boxed sequences highlight (from amino to carboxyl terminus) the first and the second histidine-rich repeats as well as the bZIP. Underlined sequences indicate the partial MafB clone (clone 17) isolated from a human fetal brain library (amino acids 148–323).

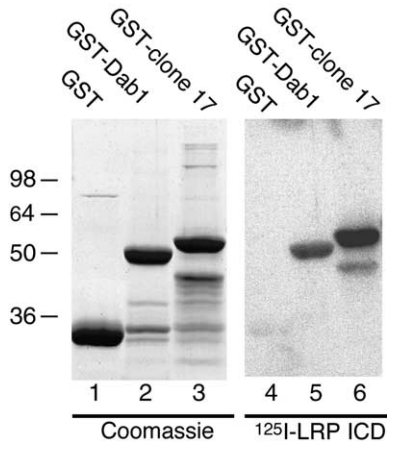


Fig. 2. Ligand blot analysis of LRPICD and clone 17 interactions. Ten microgram of purified GST (lanes 1 and 4), GST-Dab1 (lanes 2 and 5), and GST-clone 17 (lanes 3 and 6) was subjected to reducing 10% SDS–PAGE and staining with Coomassie (lanes 1–3) or transfer to nitrocellulose membrane and ligand blot analysis with ^{125}I His-LRPICD (lanes 4–6). Binding of ^{125}I His-LRPICD to GST-clone 17 and GST-Dab1, but not to GST, was detected by autoradiography.

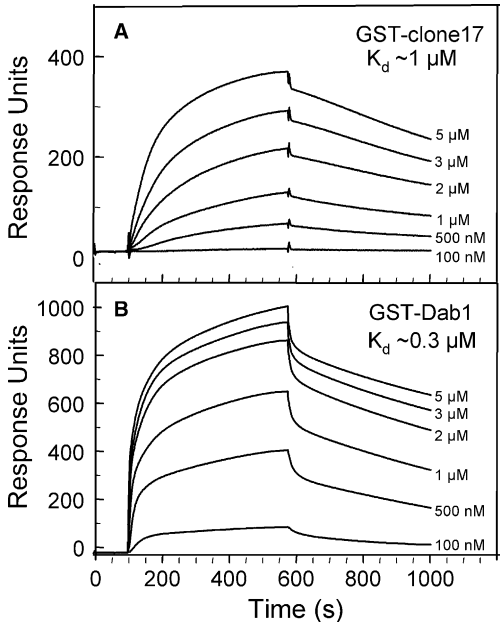


Fig. 3. Surface plasmon resonance analysis of LRPICD and clone 17 interactions. His-LRPICD immobilized on the BIAcore sensor chip surface was incubated with the indicated concentrations of purified GST-clone 17 (A) or GST-Dab1 (B). Binding to LRPICD was detected by surface plasmon resonance analysis and indicated in response units.

moter region from the Herpes simplex virus thymidine kinase promoter driving expression of the SEAP gene product. In these assays, MafB stimulated expression of SEAP as demonstrated by a 3-fold increase in enzyme activity, in accordance with previous data [22] (Fig. 5, column 2). Co-transfection of MafB and LRPICD resulted in a moderate but significant reduction of the transactivation potential of MafB (column 4), whereas LRPICD alone had no affect (column 3).

To map the binding site for LRPICD on the MafB polypeptide, we generated truncations of human clone 17 and the murine MafB sequence lacking the bZIP domain (Fig. 6A) and tested their interaction with LRPICD in the yeast two-hybrid system (Fig. 6A) and ligand blot analysis (Fig. 6B), respectively. No interaction of LRPICD with truncated gene products could be detected in both assays. Deletion of the leucine zipper but not the basic region also abolished interaction with LRPICD (data not shown), demonstrating that MafB binds to the receptor tail through its leucine zipper motif.

4. Discussion

Interactions of the LRPICD with cytosolic adaptor proteins are considered crucial for receptor function but much of the

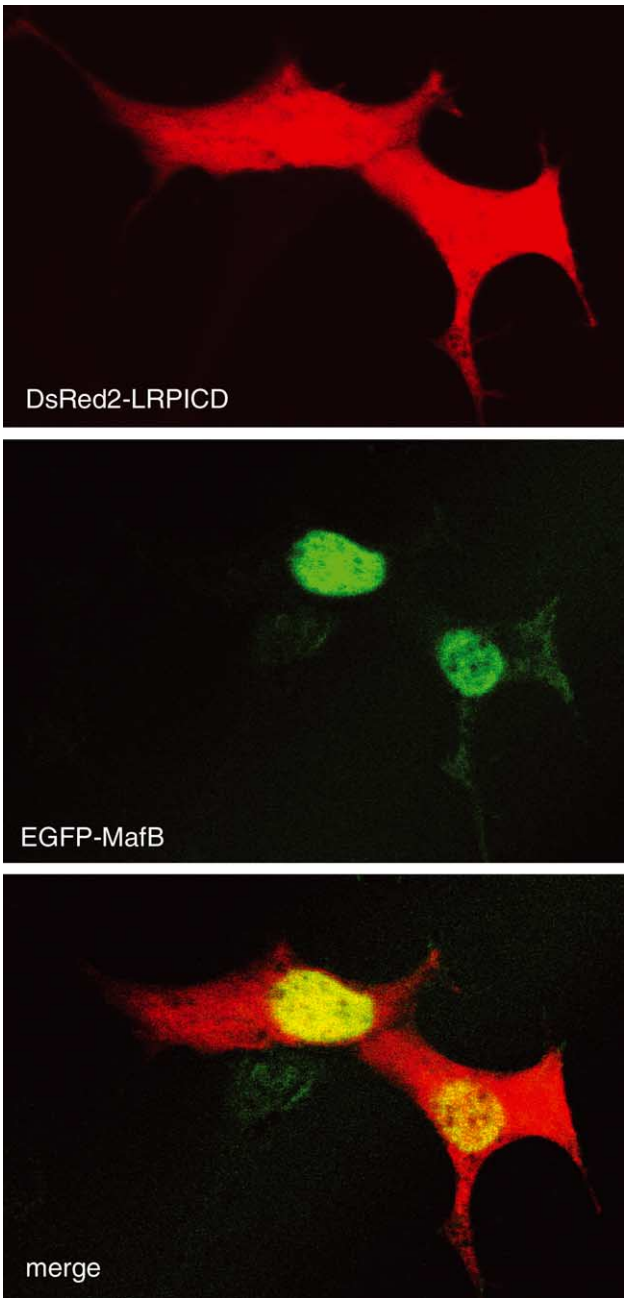
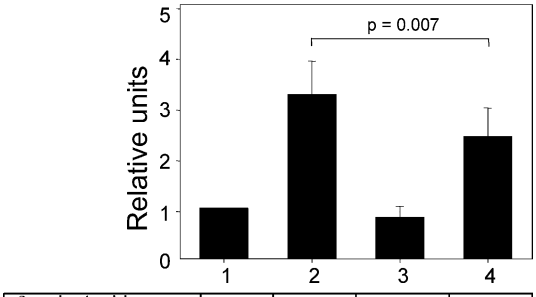


Fig. 4. Co-localization of MafB with LRPICD in HEK 293 cells. Cells were transiently transfected with constructs encoding fusions of the EGFP with MafB (EGFB-MafB) and the DsRed2 with LRPICD (DsRed2-LRPICD). Subcellular localization of the expressed proteins was detected 48 h later by confocal fluorescence microscopy.

molecular details of these interactions and the proteins involved are unclear [2,3]. Here, we report the first experimental system that enables high throughput screen for novel proteins interacting with the LRPICD, overcoming major technical obstacles encountered in conventional two-hybrid systems before [12]. Using this screen, we report the functional interaction of the LRPICD with the transcription factor MafB, suggesting a role for the receptor in transcriptional regulation of hindbrain development.

Previously, a role for the LRPICD as an independent regulatory molecule has been proposed by studies that demon-



β-galactosidase	+	+	+	+
MARE/pTALSEAP	+	+	+	+
MafB	-	+	-	+
LRPICD	-	-	+	+

Fig. 5. Effect of LRPICD on MafB activity. HEK 293 cells were transfected with reporter plasmid MARE/pTALSEAP and pCMV-β-gal (control of transfection efficiency). Where indicated, the transfection mixture also included MafB and/or LRPICD expression constructs. MafB-dependent expression of SEAP from plasmid MARE/pTALSEAP was determined and corrected for transfection efficiency as indicated by β-galactosidase activity. The relative light units obtained in the absence of MafB and LRPICD (column 1) was set at 1. A significant reduction in MafB-induced activation of the MARE/pTALSEAP was observed in the presence of LRPICD (column 4) ($P = 0.007$, Student's t-test). Data are from five independent experiments run in duplicates.

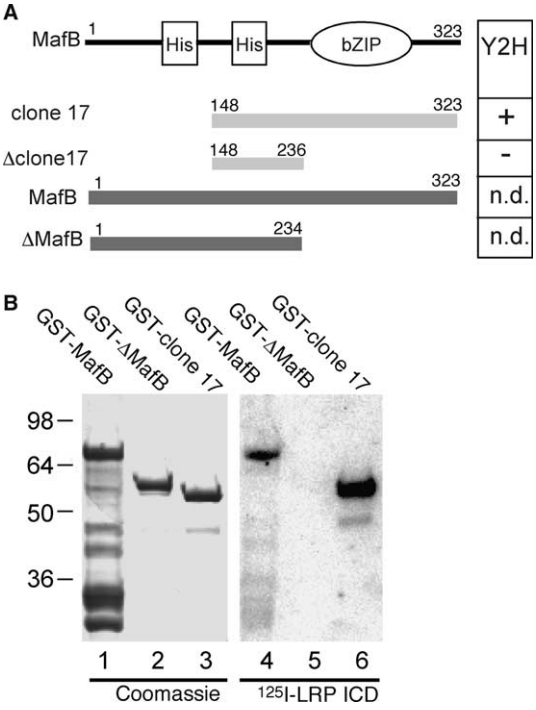


Fig. 6. Mapping of the LRPICD binding site on MafB. (A) A schematic presentation of the structure of MafB is indicated together with the extension of clone 17, truncated clone 17 (Δ clone 17), murine MafB and truncated MafB (Δ MafB). Whereas the interaction of clone 17 with LRPICD was shown in the yeast two-hybrid system, no interaction could be detected using Δ clone 17. n.d., not determined. (B) For ligand blot analysis, 10 μg of the indicated purified proteins was subjected to reducing 10% SDS–PAGE and staining with Coomassie (lanes 1–3) or transfer to nitrocellulose membranes and incubation with ^{125}I His-LRPICD (lanes 4–6). Binding of ^{125}I His-LRPICD to GST-clone 17 and GST-MafB, but not to GST-Δ MafB, was detected by autoradiography.

strated proteolytic processing of the tail by a γ-secretase-like activity [23]. Processing of the receptor tail is regulated by the glycosylation pattern of the protein and by the presence of adaptor proteins bound to it [14,23]. These findings suggest a model whereby release of the ICD from the plasma membrane may control signal transduction by translocating preformed protein complexes to other subcellular compartments. In support of this hypothesis, Kinoshita et al. [14] demonstrated trafficking of the LRPICD to the nucleus where it negatively affects transcription through a complex of the APP-derived ICD (APPICD) and the adaptor Fe65, likely by competition with APPICD for the latter.

Our findings suggest MafB as an alternative transcriptional pathway that is affected by the LRPICD. MafB belongs to a family of transcription factors that interact with other nuclear proteins to regulate gene expression during embryonic development and in cell differentiation processes. Interaction of Maf proteins with other factors is crucial for Maf protein activity, particularly because some of the Maf family members lack obvious transactivation domains [21]. For example, MafB regulates lineage specific gene expression in the hematopoietic system by interacting with Ets transcription factor, suppressing erythroid specific while stimulating monocyte specific differentiation processes [24,25].

Given the embryonic lethality observed in LRP knockout mice [10], an established role for MafB in embryonic development may be of particular relevance when considering interaction of both proteins. MafB is the product of the murine *Kreisler* gene, a recessive mutation that affects hindbrain segmentation [20]. Based on the findings obtained in HEK293 cells (Fig. 5), one may postulate a negative influence of LRPICD on MafB transcription factor activity. The significant but modest inhibitory effect of LRPICD on MafB activity observed in this cell system may reflect the absence of additional co-factors that are required for this protein interaction network and that may be expressed in a cell type specific manner as shown for the hematopoietic system [24,25].

In conclusion, our studies have established a novel screening system for proteins interacting with LRPICD that overcomes limitations encountered in previous approaches and that will significantly facilitate the elucidation of the various receptor functions. Furthermore, identification of MafB as LRPICD interacting protein supports a role of the tail domain as independent regulator of transcription in the nucleus and highlights possible mechanisms responsible for embryonic lethality of the LRP1 knockout mouse model.

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