Haploinsufficiency of RREB1 causes a Noonan-like RASopathy via epigenetic reprogramming of RAS-MAPK pathway genes

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RAS-MAPK signaling mediates processes critical to normal development including cell proliferation, survival, and differentiation. Germline mutation of RAS-MAPK genes lead to the Noonan-spectrum of syndromes. Here, we present a patient affected by a 6p-interstitial microdeletion with unknown underlying molecular etiology. Examination of 6p-interstitial microdeletion cases reveals shared clinical features consistent with Noonan-spectrum disorders including short stature, facial dysmorphia and cardiovascular abnormalities. We find the RAS-responsive element binding protein-1 (RREB1) is the common deleted gene in multiple 6p-interstitial microdeletion cases. Rreb1 hemizygous mice display orbital hypertelorism and cardiac hypertrophy phenocopying the human syndrome. Rreb1 haploinsufficiency leads to sensitization of MAPK signaling. Rreb1 recruits Sin3a and Kdm1a to control H3K4 methylation at MAPK pathway gene promoters. Haploinsufficiency of SIN3A and mutations in KDM1A cause syndromes similar to RREB1 haploinsufficiency suggesting genetic perturbation of the RREB1-SIN3A-KDM1A complex represents a new category of RASopathy-like syndromes arising through epigenetic reprogramming of MAPK pathway genes.
The MAPK pathway is an essential signaling cascade that controls cell proliferation, cell survival, motility and differentiation all of which are perturbed in cancer but are also critical to normal development\(^1\). Activation of RAS, encoded by one of three isoforms HRAS, KRAS, or NRAS, is coupled to the transduction of mitogenic signals from growth factor receptors to multiple downstream effector pathways including RAF-MEK-ERK (MAPK) to regulate transcription\(^2-3\). The activation of the MAPK cascade involves the sequential phosphorylation of serine/threonine kinases RAF, MEK1/2 and ERK1/2\(^4\). MAPK pathway regulation and controlled expression of MAPK target genes is essential in mediating diverse physiologic outcomes including cellular transformation, tumorigenesis, developmental disorders, cardiac hypertrophy and heart failure.

Germline gain-of- and loss-of-function mutations in genes of the RAS-MAPK pathway lead to the Noonan-spectrum of autosomal dominant disorders, a group of malformation syndromes affecting 1 in 1000 individuals\(^5-7\). Noonan-spectrum, including Noonan-syndrome (NS), Costello and cardio-facio-cutaneous (CFC) syndromes, and others are collectively referred to as RASopathies, display an overlap of clinical features including developmental delay, intellectual disability, craniofacial dysmorphism, and cardiac defects\(^8-9\). The characteristic craniofacial abnormalities include wide-set eyes, broad forehead, wide nasal base, and downward slanting palpebral fissures\(^9\). Cardiac hypertrophy and abnormalities are common in the RASopathies\(^10,11\) and NS is a leading cause of congenital heart disease\(^12\). Germline missense mutations have been identified in PTPN11, KRAS (NS), HRAS (Costello syndrome), and MAP2K1/2 (CFC syndrome)\(^7\). Although significant efforts have been made in identifying causative genes of NS and NS-like disorders, the underlying genetic causes for up to 20% of NS-like cases are unknown suggesting additional mechanisms of causation\(^13\).

Here, we present a patient with an interstitial 6p25.1p24.3 microdeletion and demonstrate that shared clinical features of previously identified 6p interstitial microdeletion cases\(^14-18\) significantly overlap with the Noonan-spectrum of disorders. The consequences of haploinsufficiency of genes within the 6p25.1p24.3 region remain to be defined. We identify the RAS-effector, RAS responsive element binding protein 1 (RREB1) as the gene commonly lost in 6p interstitial microdeletion cases for which there is genomic data. RREB1 is a large zinc-finger transcription factor (TF) implicated in RAS signaling and cancer\(^19\). RREB1 regulates transcription by binding to RAS-response elements (RRE) in target promoters downstream of RAS-MAPK pathway activity\(^19-23\). The clinical relevance of RREB1 expression, the target genes RREB1 controls and its role in regulating the RAS-MAPK pathway in human tissues is poorly understood. The mechanism by which RREB1 regulates transcription remains unknown. We find Rreb1 hemizygous mice display orbital hypertelorism and age dependent cardiac hypertrophy phenocopying the human 6p25.1p24.3 syndrome. Rreb1 haploinsufficiency resembles a RASopathy-like syndrome and leads to sensitization of MAPK signaling in fibroblast and cardiac cells. RREB1 recruits SIN3A and KDM1A to an RRE in target promoters in human and murine cells to control histone H3K4 methylation of MAPK pathway genes.

**Results**

**RREB1 haploinsufficiency causes a RASopathy-like malformation syndrome.** We identified an eight-year-old boy who exhibited short stature, mild intellectual disability, developmental delay and widely spaced eyes (Fig. 1a) (complete case report in Supplementary Note 1). The facial appearance and other features were reminiscent of a Noonan-spectrum (NS) disorder. Diagnosis of NS-like syndrome was explored by whole exome sequencing but no pathogenic variants in 13 known NS genes were identified. A chromosomal array analysis revealed a heterozygous 2.1–2.7 Mb interstitial microdeletion of 6p25.1p24.3 which contained 11 genes (Fig. 1b).

Examination of phenotypes associated with 6p interstitial microdeletion cases referenced in the literature\(^14-18\) and cases reported in the NCBI-ClinVar database with copy number loss involving 6p25.1p24.3 display an overlap of clinical features including intellectual disability, craniofacial dysmorphism, and cardiac abnormalities (Fig. 1c, Supplementary Fig. 1a). Examination of the break points reported for these fourteen 6p interstitial microdeletion cases revealed the shortest region of overlap which contained only the RREB1 gene (Fig. 1d).

We examined RREB1 expression in EBV-transformed lymphoblastoid cells (LCLs) derived from the proband and confirmed reduction of RREB1 mRNA and protein in the proband (\(p<0.01\) t-test, Fig. 1e, Supplementary Fig. 1b). We validated two additional genes contained within the deleted region, SSR1 and LYZ6, and found they were similarly decreased in LCLs from the proband compared to parents (Supplementary Fig. 1b, c). LCLs derived from the proband had significantly increased phosphorylated (p)-MEK and p-ERK when stimulated with FBS with increased proliferation compared to LCLs derived from either parent (\(p<0.0001\) t-test, Fig. 1f, g and Supplementary Fig. 1d, e) consistent with aberrant RAS-MAPK pathway activity associated with a RASopathy-like syndrome.

To model RREB1 haploinsufficiency as the driver of a malformation syndrome, we generated a Rreb1 knockout mouse model. We used CRISPR-Cas9 to disrupt the coding frame of murine Rreb1 in C57BL6 zygotes (Fig. 2a, Supplementary Fig. 2a). Heterozygous (Rreb1+/−) progeny were obtained but following heterozygous crosses no Rreb1−/− mice were obtained. Examination of the uterus from pregnant females revealed multiple implantation sites with no embryonic remains suggesting Rreb1−/− embryos died after implantation prior to E9.5 (Supplementary Fig. 2b)\(^24\). Thus, homozygous Rreb1 deletion is lethal during early embryogenesis, whereas one copy of the Rreb1 allele is sufficient for postnatal viability. Rreb1+/− mice were slightly smaller with a wider blunted nose and significantly increased intercanthal distance (ICD, \(p<0.001\) t-test) compared to WT littermates (Fig. 2b–d and Supplementary Fig. 2c, d) similar to the changes in the cranial bones observed in the Noonan syndrome mouse model\(^25\).

Since cardiac hypertrophy and dysfunction are commonly observed in patients with 6p microdeletion and Noonan-like syndromes\(^7,9,14-18\), the hearts from Rreb1+/− and WT littermates were compared. Rreb1 mRNA was expressed at approximately half in Rreb1+/− hearts (Supplementary Fig. 2e). Rreb1+/− mice developed left ventricular hypertrophy at 6 months of age with pronounced cardiac wall thickening (Fig. 2e). The cross-sectional area of cardiomyocytes was more than 2-fold higher (\(p<0.001\) t-test) in Rreb1+/− mice compared with WT and also present in younger animals (Fig. 2f, g and Supplementary Fig. 2f). The heart weight to tibial length of Rreb1+/− mice revealed significantly smaller normalized heart size (\(p<0.01\) t-test) when compared to WT littermates at 6 months of age (Supplementary Fig. 2g). Analysis of cardiac performance by echocardiography revealed an age related decreased fractional shortening in Rreb1+/− hearts at 6 months of age (\(p<0.001\) t-test) but not in younger mice (Fig. 2h, Supplementary Fig. 2h, i). Transcripts for the atrial/brain natriuretic factors (ANP, BNP) and β-myosin heavy chain (β-MHC), canonical markers of the fetal gene program and cardiac hypertrophy were up-regulated in Rreb1+/− hearts at 6 months (Supplementary Fig. 2j). RAS-dependent hypertrophic heart
RREB1 is a negative regulator of RAS-MAPK pathway target genes. To ascertain the molecular mechanism underlying the negative regulation of the MAPK pathway by RREB1 haploinsufficiency, we performed RREB1 chromatin immunoprecipitation followed by sequencing (ChIP-seq) in HEK293 cells. Since ChIP grade antibodies are not available for RREB1, we created a tetracycline inducible flag-tagged RREB1 cell line for ChIP experiments. Treatment of cells with tetracycline activated RREB1 mRNA expression 10-fold over untreated cells with RREB1 protein over-expression detected at physiologic levels (Supplementary Fig. 3a, b). ChIP-seq revealed 1239 unique broad peaks and 7488 sharp peaks associated with 3726 genes. RREB1 binding was enriched primarily at promoters centered on the transcription start site (TSS) followed by intronic and distal regions (Fig. 3a, Supplementary Fig. 3c). KEGG analysis revealed RREB1 target genes enriched in metabolic pathways, MAPK signaling and actin cytoskeleton (Fig. 3b).

To support the ChIP-seq results, we performed RNA-seq in RREB1 knockdown cells. HEK293 cells expressing a short-hairpin RNA (sh1) targeting RREB1 had 80% reduction in RREB1 mRNA and 50% reduction in RREB1 protein compared to control (Supplementary Fig. 3d, e). By RNA-seq analysis, 1457 mRNAs had greater than 2-fold differential expression changes and 87% were upregulated with RREB1 knockdown. KEGG analysis of RREB1–transcriptome confirmed enriched MAPK pathway target genes which included the small GTPase HRAS, multiple MAP3Ks including MAP2K2 (MEK2), signaling molecules (AKT1, DUSP7, FGFR4) and transcription factors (JUN, JUND, MYC) (Fig. 3c, Supplementary Fig. 3f). RREB1 ChIP-seq corroborated regulation of MAPK pathway genes and revealed RREB1 occupancy proximal to a RAS-response element (RRE) on the promoters of the FGFR4, HRAS, and MAP2K2 gene loci (Fig. 3d).

RREB1 binding was confirmed on the JUN, MYC, FGFR4, HRAS and MAP2K2 promoters by ChIP (Fig. 3e). RREB1 binding was not detected on the JUND or AKT1 promoters suggesting dysregulation of these genes was an indirect consequence of RREB1 knockdown (Supplementary Fig. 3g). Previously identified RREB1 targets miR-143/145 (MIR143/145) and ARHGEP2 were used as positive controls for ChIP and corroborated previous results (Fig. 3e, Supplementary Fig. 3g).

To demonstrate the direct regulation of target genes by RREB1, we cloned proximal promoters of FGFR4, HRAS and MAP2K2 into the pGL3-luciferase reporter and conducted promoter activation assays in cells with RREB1 knockdown (Fig. 3f). Promoter activity of all three genes was higher in HEK293 cells

disease in mice is histologically indistinguishable from hypertrophic cardiomyopathy in humans and is a characteristic Noonan-like phenotype associated with gain-of-function MAPK signaling. Consistent with cardiac hypertrophy observed in Rrebl+/− mice and sensitization of MAPK signaling in RREB1 haploinsufficient cells, increased p-MEK and p-ERK was observed in left ventricle (LV) from Rrebl+/− hearts compared to WT (Fig. 2i).

We also examined MAPK signaling as a consequence of Rrebl homozygosity in murine embryonic fibroblasts (MEFs) derived from the proband (Boy) and parents (P1, P2; n = 6). Four independent experiments, p = 0.0002 (P2 vs. Boy) two-tailed student’s t-test, box plots indicate the IQR of the data and the central line shows the median. Western blot analysis of MAPK signaling in LCLs (P1, P2, Boy) following serum deprivation and stimulation with FBS for the indicated times. Experiment was initially a single membrane (provided in Supplementary Fig. 1), separated post exposure for clarity in the figure. Tubulin served as a loading control. Blots are representative of n = 6 experiments.

Relative growth (arbitrary units) of LCLs (P1, P2, Boy) in culture measured with Alomar Blue. Data average of n = 6 independent experiments, p = 3.4E-08 (P1 vs. Boy), p = 6.3E-10 (P2 vs. Boy) two-tailed student’s t-test, error bars presented as mean values ± SD. Source data are provided as a Source Data file.
expressing sh1-RREB1 or a second shRNA targeting RREB1 (sh2) compared to sh-control demonstrated that negative regulation of promoter activity was relieved by RREB1 knockdown. MAPK genes JUN, MYC, FGFR4, HRAS and MAP2K2 identified by RNA-seq were upregulated at both the mRNA and protein levels in HEK293 cells expressing sh1 or sh2 targeting RREB1 compared to control (Supplementary Fig. 3h, i). Consistent with a mechanism of RREB1 repression of target genes, HEK293 cells with the tetracycline inducible flag-tagged RREB1 had repressed levels of FGFR4, HRAS and MAP2K2 mRNAs when cells were treated with tetracycline to induce RREB1 expression (Fig. 3g). Importantly, HRAS and MAP2K2 transcript and protein levels were elevated in LCLs derived from the proband compared to either parent consistent with the conclusion that these are RREB1 dysregulated genes in the patient (Supplementary Fig. 3j, k). FGFR4 mRNA was not detectable in the LCLs. Increased expression of Fgfr4, Hras and Map2k2 mRNAs was observed in Rreb1 deficient fibroblasts and cardiac cells (Fig. 3h, i). Luciferase expression driven by human FGFR4, HRAS and MAP2K2 promoters was increased in Rreb1+/− MEFs relative to WT demonstrating transcriptional repression mediated by Rreb1 in murine cells (Supplementary Fig. 3l). Endogenous examination of transcripts revealed Hras upregulated in Rreb1+/− hearts from 3 month old animals which remained high in hearts examined at 6 months while Fgfr4 and Map2k2 expression increased in Rreb1+/− hearts as animals aged (Fig. 3j, Supplementary Fig. 3m). The proteins encoded by Fgfr4, Hras and Map2k2 (Mek2) were similarly upregulated in Rreb1+/− hearts (Fig. 3k). Fgfr4 expression was pronounced in LV sections of 6 month Rreb1+/− hearts in comparison to WT (Supplementary Fig. 3n). These data provide direct evidence that RREB1 negatively regulates the transcription of key MAPK components and identifies Fgfr4, Hras.
**Fig. 3** RREB1 transcriptionally regulates MAPK pathway target genes. **a** Genomic distribution of RREB1 ChIP-seq peaks. Distal (intergenic), UTR (5’ and 3’-UTR), downstream (next to annotated genes). **b** KEGG terms associated with RREB1 target genes identified by ChIP-seq. **c** Heat map view of KEGG defined MAPK pathway genes differentially expressed by RNA-seq analysis in HEK293 cells expressing control shRNA (Con) versus shRNA targeting RREB1 (sh1). Color scale ranges from yellow lowest expression to black highest expression. **d** Gene track view of RREB1-ChIP-seq peaks at the indicated loci. Gene bodies are shown above the track sets. Black arrows of the gene bodies mark the transcription start site. Red arrows point to the RAS response element. **e** RREB1 ChIP-PCR in HEK293 cells with a tet-inducible RREB1 transgene treated with DMSO (low RREB1) or tetracycline to induce RREB1 expression (high RREB1). Amplicons for the indicated genes were queried around promoters (P) designed 100–500 bp from the TSS (inset). For this and subsequent ChIP experiments, enrichment is relative to IgG (n = 3). Error bars presented as mean values ± SD. **f** Upper panels depict gene track view of H3K27 acetylation (ENCODE Regulation, Layered H3K27ac track) proximal to the indicated promoters. Red arrows mark the putative RRE. Bar graphs show normalized luciferase activity (arbitrary units) driven from pGL3-reporter constructs with the indicated promoters (gray bars in the upper panels) in HEK293 cells expressing control shRNA (Con) or shRNA (sh1 or sh2) targeting RREB1 (n = 3; **p < 0.001, ***p < 0.0001 two-tailed student’s t-test, error bars presented as mean values ± SD). **g** RT-QPCR analysis of the indicated genes in HEK293 cells with tet-inducible RREB1 transgene described in panel d (n = 4, **p < 0.01, ***p < 0.001 two-tailed student’s t-test). **h** Expression of the indicated mRNAs in WT and Rreb1−/− MEFS (n = 6) and i expression in WT and Rreb1−/− hearts from 6 month old mice (n = 5) (**p < 0.01, ***p < 0.001, ****p < 0.0001 two-tailed student’s t-test). Box plots (panels g,h,i) indicate the IQR of the data and the central line shows the median. **j** Analysis of the indicated proteins in WT and Rreb1+/− hearts lysates derived from 6 month old mice. (n = 5). Gapdh served as a loading control. Source data are provided as a Source Data file.

RREB1 negatively regulates FGF-HRAS-MAPK signaling. Since MAPK pathway genes were dysregulated in the RREB1 transcriptome, we hypothesized RREB1 knockdown sensitized cells to RAS-MAPK signaling. In shRNA-RREB1 cells, stimulation with FBS increased p-MEK and p-ERK compared to sh-control cells (Fig. 4a, Supplementary Fig. 4a). No change in AKT signaling was observed even though AKT levels were visibly higher with RREB1 knockdown. RREB1 knockdown cells proliferated significantly faster (p < 0.001 t-test) and formed larger colonies in soft agar consistent with elevated MAPK signaling (Fig. 4b, c). Multiple genes are commonly lost in the 6p25.1p24.3 microdeletion cases; therefore we wanted to demonstrate that decreased expression of RREB1 rather than decreased expression of neighboring genes caused sensitization of MAPK signaling. We examined the expression of the nearest neighbor genes to RREB1 in HEK293 cells and found they only express SSR1 in addition to RREB1 (Supplementary Fig. 4b). Targeting SSR1 using two independent shRNAs had no effect on p-MEK and p-ERK compared to sh-control cells (Supplementary Fig. 4c, d) confirming a RREB1 specific role for MAPK pathway regulation.

Next, we examined MAPK pathway activation in response to specific mitogens or cytokines in RREB1 knockdown cells (Fig. 4d). Treatment with EGF which activates the epidermal growth factor receptor (EGFR), FGF1 or FGF2 which activates FGF receptors (1–4) increased p-ERK in sh1-RREB1 cells compared to control. FGF16, a ligand specific for FGFR4, strongly activated p-ERK in sh1-RREB1 cells greater than 2-times compared to control cells. In distinction, FGFR treatment, which specifically activates FGFR3, had a modest effect on sh1-RREB1 cells. HGF (hepatocyte growth factor) which activates the receptor tyrosine kinases (RTK) c-MET also increased p-ERK in sh1-RREB1 cells compared to control. Treatment with TNF-α, a minimal ERK activator or TGF-β, which does not activate ERK, had no enhancing effect in sh-RREB1 cells. Therefore, RREB1...
negatively regulates MAPK signaling specifically downstream of EGFR, FGFR4 and MET-RTKs.

Since FGFR4 and RTKs activate the canonical RAS-MAPK pathway, we examined the RAS isoform principally activated in RREB1 deficient cells. We affinity-purified RAS family members bound to a Raf derived Ras-binding domain fused to GST (Fig. 4e) and observed elevated total RAS-GTP in sh1-RREB1 cells compared to control (Supplementary Fig. 4e). Using isocitrate-specific RAS antibodies the elevated RAS activity was attributed to increased activation of HRAS (p < 0.02, Fig. 4f, Supplementary Fig. 4e). This result is consistent with increased gene dosage of HRAS mRNA and protein levels resulting in increased HRAS activation. Therefore, we tested the importance of HRAS expression in sensitizing MAPK pathway activation. Using cell lines expressing shRNA targeting RREB1 or control, we depleted HRAS with siRNA to transiently block HRAS signaling and observed decreased p-ERK in sh-RREB1 cells (Fig. 4g, Supplementary Fig. 4f). Similarly, we tested whether increased MAP2K2 expression was sufficient to increase MAPK signaling. HEK293 cells transfected with a MAP2K2-myc tagged transgene had 2-fold more MEK2 (encoded by the MAP2K2 gene) and increased p-ERK when stimulated with FBS compared to cells expressing the empty-vector (Fig. 4h, Supplementary Fig. 4g, h). These data show that transcriptional dysregulation of FGFR4, HRAS and MAP2K2 as a result of RREB1 haploinsufficiency is both necessary and sufficient to functionally activate MAPK pathway signaling in different cell types.

RREB1 interacts with epigenetic chromatin regulation machinery. Since RREB1 lacks an intrinsic transcription activation domain, the mechanism of RREB1 transcriptional regulation is likely to occur through recruitment of other factors21. To understand how RREB1 mediates transcriptional regulation, we conducted RREB1-BioID in the HEK293 model system28,29. We identified 241 high-confidence RREB1-interacting proteins using this methodology (1% FDR). The RREB1-interactome comprised sequence specific TFs, transcriptional repressors/activators, and chromatin remodeling proteins (Fig. 5a, Supplementary Fig. 5a). All known members of the CtBP1 complex31 were detected including 7 high-confidence interactors. The RREB1-interactome was composed of transcriptional and chromatin regulators and GO-molecular function identified histone demethylases, regulators of H3K4 methylation and proteins involved in heart development (Supplementary Fig. 5a–c). We validated the interaction between RREB1 and several components using bimolecular fluorescence complementation (BiFC)32. Consistent with BioID, intense nuclear fluorescence was observed when cells expressed VC-RREB1 and Myc-tagged Mek2 (+Mek2) following stimulation with FBS at the indicated times (n = 4). Tubulin served as a loading control. Source data are provided as a Source Data file.
H3K4me3, K4me2, K36me3 and K9ac in lysates derived from sh1-RREB1 and Rreb1+/− MEFs compared to control cells (Fig. 5b, c). Increased H3K4me2 and K4me3 were observed in lysates from Rreb1+/− hearts compared to WT (Fig. 5c).

We conjectured that RREB1 loss is associated with increased H3K4me2/3 marks on FGFR4, HRAS, and MAP2K2 promoters. We examined H3K4 modification at these target loci and found increased K4me2 and K4me3 at the FGFR4, HRAS, and MAP2K2 promoters in cells expressing sh1-RREB1 compared to control (Fig. 5d). Consistent with ChIP, H3K4me2 proximal to an RRE is prominent at these promoters mapped in A562 cells by ENCODE (Fig. 5d). H3K4me2 and K4me3 were enriched on promoters near a putative RRE in 8-week old murine hearts (Supplementary Fig. 5e) demonstrating these promoters are active during heart development. We found increased H3K4me2 and K4me3 at all three promoters in Rreb1+/− hearts compared to WT (Fig. 5f). These data substantiate that loss of RREB1 results in epigenetic remodeling associated with transcriptional activity at MAPK pathway genes.

RREB1 recruits SIN3A and KDM1A to promoters. We identified a novel interaction between RREB1 and the transcriptional repressor SIN3A (switch-insensitive 3 family member A) with RREB1-BioID. SIN3A haploinsufficiency has been described displaying intellectual disability, developmental delay, facial dysmorphism and short stature features similar to the phenotypes of 6p interstitial microdeletion suggesting RREB1 and SIN3A may be part of a common genetic pathway. GO-molecular function analysis of the RREB1-interactome revealed a potential transcriptional repressor complex that included SIN3A, histone demethylases and other proteins (Supplementary Fig. 6a). SIN3A functions as a scaffold with no DNA binding domain or enzymatic activity. We hypothesized that the DNA binding function of RREB1 recruits SIN3A and associated proteins to target promoters to regulate histone modifications. Supporting this idea, greater than 70% of the RREB1 ChIP-seq peaks overlapped with SIN3A ChIP-seq and KEGG analysis of the overlap revealed a MAPK signaling signature (Supplementary Fig. 6b).

To define components in a RREB1-SIN3A complex, we precipitated a binary complex that contained VC-RREB1 and VN-SIN3A with an antibody that recognizes the fully complemented Venus (VC + VN) for analysis by mass spectrometry.
(IP-MS) (Fig. 6a, Supplementary Fig. 6c). We used BioPlex (biophysical interactions of ORFeome-based complexes) Network 2.0 to analyze the IP-MS dataset against validated protein-protein interactions. Using this approach, we discovered a minimal RREB1-SIN3A complex (Fig. 6b) that contained SIN3A and associated factors and known components of a CoREST complex. In addition, the RREB1-SIN3A complex was coupled to proteins involved in RNA splicing, RNA end formation, and negative regulation of transcription thus connecting downstream RNA processes to the activity of the RREB1-SIN3A complex (Supplementary Fig. 6d).

To validate the existence of an endogenous RREB1-SIN3A complex, we performed glycerol gradient ultracentrifugation of lysates derived from HEK293 cells followed by western blot analysis of isolated fractions. We observed that all three isoforms of endogenous RREB1 (three human isoforms MW 188, 181 and 159 KDa) co-eluted in the same fractions with SIN3A, RBBP7, HDAC1/2, KDM1A and RCOR1 consistent with a common macromolecular complex (Fig. 6c). Notably, RREB1-SIN3A eluted earlier from CTBP1 suggesting a distinct complex from RREB1-CTBP1. CTBP1 appeared to be predominantly associated with the 159 KDa isoform of RREB1 (Fig. 6c).

Recently, KDM1A has been shown to be part of a SIN3A-HDAC complex and is functionally associated with the same promoters. In a reciprocal BioID screen, we identified RREB1 as a high confidence interactor using KDM1A as the bait (Supplementary Fig. 6e). We hypothesized that RREB1 target gene regulation depended on the recruitment of SIN3A-KDM1A to promoters. We knocked down SIN3A or KDM1A in HEK293 cells and observed increased FGFR4 and HRAS mRNA in cells expressing siRNA targeting SIN3A and increased HRAS and MAP2K2 mRNA in cells expressing siRNA targeting KDM1A (Fig. 6d, Supplementary Fig. 6f). Dysregulated expression of these genes in either SIN3A and/or KDM1A knockdown cells recapitulated sensitization of MAPK pathway signaling we...
observed in RREB1 haploinsufficient cells providing evidence that modification of H3K4 methylation is sufficient to amplify MAPK pathway signaling (Supplementary Fig. 6f–h).

To establish that recruitment of SIN3A-KDM1A to promoters was dependent on RREB1, we performed SIN3A and KDM1A ChIP in HEK293 cells using Tet inducible RREB1. SIN3A and KDM1A were found in proximity to an RRE in the FGFR4, HRAS and MAP2K2 promoters mapped in MCF-7 cells (SIN3A) or K362 cells (KDM1A) by ENCODE (Fig. 6e). In cells with high RREB1 expression, SIN3A ChIP was enriched on the FGFR4 and HRAS promoters and KDM1A ChIP was enriched on the HRAS and MAP2K2 promoters (Fig. 6e). Cells with high RREB1 expression had decreased H3K4me2 on all three promoters compared to control cells (Fig. 6e), consistent with KDM1A as a histone demethylase for H3K4me1/2.

In murine erythroleukemia cells, Sin3a binding has been mapped to the Fgf4, Hras and Map2k2 promoters (Fig. 6f). We performed ChIP in Rreb1+/− and WT MEFs and hearts to determine how Rreb1 haploinsufficiency affected occupancy of murine Sin3a and Kdm1a. Rreb1+/− MEFs had decreased Sin3a on Fgf4, Hras and Map2k2 promoters and decreased Kdm1a on the Hras and Map2k2 promoters compared to WT cells (Fig. 6g). Decreased Sin3a and Kdm1a occupancy was observed on the Fgf4, Hras and Map2k2 promoters in Rreb1+/− hearts compared to WT (Fig. 6h). Therefore, MAPK pathway genes are regulated by Rreb1-Sin3a-Kdm1a in murine fibroblasts and cardiac cells.

Discussion

Our data demonstrate that RREB1 forms a transcriptional repressive complex together with SIN3A and KDM1A which normally leads to transcriptional inactivation of MAPK signaling components. Single allele loss of RREB1 leads to functional loss of lysine demethylase activity at RREB1 target promoters and hence increased H3K4me2/3 marks leading to transcriptional repression and MAPK pathway activation. We provide genetic and biochemical evidence that dysregulation of Fgf4, Hras and Map2k2 in the heart of Rreb1 heterozygous mice is deleterious leading to a cardiomyopathy that is reminiscent of the phenotype observed in Noonan-like RASopathies. Therefore, we propose that RREB1 is a new RASopathy gene that triggers MAPK pathway activation through the loss of epigenetic repressive marks on critical proteins involved in MAPK pathway signaling.

Genetic disorders caused by haploinsufficiency of transcriptional machinery and epigenetic regulators are emerging as drivers of developmental syndromes. We find that RREB1 haploinsufficiency resembles a RASopathy in both overlap of clinical features and sensitization of RAS-MAPK signaling observed in multiple cell types. In distinction to the known RASopathies, which are single gene disorders, the 6p25.1p24.3 microdeletion syndrome arises via transcriptional dysregulation of multiple RREB1 target genes including FGF4, HRAS and MAP2K2 that result in sensitization of the MAPK pathway to activation. The germline gain-of-function mutations in HRAS and MAP2K2 associated with Costello syndrome and CFC syndrome, respectively, argue that the increased transcription of RREB1 targets HRAS and MAP2K2 observed with single gene loss of RREB1 account for the overlapping phenotypes observed in patients with these syndromes and in the Rreb+/− hemizygous in mice.

Rreb+/− hemizygous in mice are phenotypically similar to the Noonan mouse model observed with gain-of-function mutation in Ptpn11. These characteristics include smaller stature, cranial facial dysmorphism and cardiac abnormalities. Mouse models of other RASopathies have been developed including introduction of a germline G12V mutation in the endogenous Hras locus which phenocopied some abnormalities observed in patients with Costello syndrome, including facial dysmorphism and cardiomyopathies. The Costello mice displayed systemic hypertension, vascular remodeling, and fibrosis in the heart which was age dependent and a consequence of abnormal up regulation of the renin–Ang II system. We have observed an age dependent mechanism of cardiac dysfunction in Rreb+/− mice. Future studies will address additional phenotypes such as vascular remodeling and the potential alteration of the renin-Ang II system. A mouse model of CFC syndrome with gain-of-function Braf mutation leads to craniofacial malformations, congenital heart defects, musculoskeletal abnormalities and growth delay. A mouse model of CFC syndrome harboring a Map2k2 mutation is yet to be reported. However, we postulate that the similarities in phenotypes between available RASopathy mouse models and the Rreb1+/− mice highlight the deleterious effect of overactive RAS-MAPK signaling on organismal development.

Our results show that SIN3A and KDM1A are components of a RREB1 repressor complex and each component contributes to sensitization of MAPK signaling. Haploinsufficiency of SIN3A and KDM1A mutations lead to similar syndromes characterized by the clinical features observed with RREB1 haploinsufficiency including developmental delay and craniofacial abnormalities. Microdeletion of 15q24 encompassing the SIN3A gene and point mutations in SIN3A share striking features that include facial dysmorphism, short stature and mild intellectual disability and link SIN3A loss of function to 15q24 microdeletion syndrome. De novo mutations in the KDM1A allele have been identified in three individuals who share similar clinical features including facial features, global developmental delay and hypotonia. We propose that RREB1, SIN3A and KDM1A are part of a common genetic pathway that defines a new spectrum of RASopathy-like disorders linked to the genetic perturbation of components of this newly defined silencing complex.

Deletion of the terminal end of chromosome 6 is a clinically recognized syndrome called 6pter-p24 deletion syndrome characterized by developmental delay, cardiac abnormalities and craniofacial dysmorphism thought to be caused by haploinsufficiency of the transcription factor FOXC1. Terminal deletion of 6p with breakpoints in 6p25.3 represents the vast majority of cases. These syndromes are recognized as distinct from branchiooculofacialis syndrome (BOFS) caused by haploinsufficiency or mutation of the transcription factor AP-2α encoded by TFAP2A on the centromeric end of 6p24.3. Clinical cases with interstitial microdeletion of chromosome 6 in the 6p25.1p24.3 region not involving FOXC1 or TFAP2A display an overlap of clinical features with 6pter-p24 deletion syndrome hinting at a distinct etiology. Interestingly, FOXC1 and TFAP2A were both found in proximity to RREB1 using BioID suggesting some target genes may be co-regulated by these transcription factors.

In conclusion, we have provided mechanistic evidence that RREB1 haploinsufficiency is the pathogenic cause underlying the clinical phenotypes observed in the 6p25.1p24.3 microdeletion syndromes. The clinical features of 6p25.1p24.3 microdeletion cases as well as the physiological and biochemical consequences of RREB1 deficiency described in our study define a novel mechanism for sensitization of MAPK signaling and the development of a Noonan-like RASopathy disorder. RREB1 haploinsufficiency thus represents a new category of RASopathy-like syndromes arising through transcriptional overexpression of MAPK pathway genes as a result of deregulated epigenetic control.
5.0 × 10^3 cells plated in 96-well plate (n = 8) grown for 24–72 h and treated with Anaclick Blue (Thermofisher) according to the manufacturer’s protocol.

ChIP assays were performed with HEK293 cells using antibody to RREB1 and ChIP-seq analysis using the genome browser tool, genome browser tool analysis, and compared to previously published datasets.

**Gene expression analysis.** Total RNA was isolated from cells with TRIzol (Invitrogen) according to the manufacturer’s protocol. cDNAs were made using the Quantitect kit (Qiagen). QPCR was performed using an ABI Step One-Plus System with LUNA Universal master mix (NEB).

**ChIP-seq analysis.** Raw reads were aligned to hg38 using bwa (version 0.7.15) (PMID: 22569178). The resulted sam files are converted to bam with samtools (version 1.8) (PMID: 21245279). MACS2 (version 2.1.0) (PMID: 18793982) was used to call peak on the bam files. bedGraph files containing signal per million reads produced from MACS2 was converted to bigwig files with ucsc tool kit (315). Selected publicly-available ChIP-seq datasets (GSM803530, GSM91830, GSM803525 and GSE91601) were obtained from the Gene Expression Omnibus (GEO). The results were parsed for genes of interest, the data subsetted and converted to bedGraph format using tools available from the UCSC Genome Browser. Data was imported into R using established packages. Processed ENCODE tracks were filtered for significant peaks based on matched p-value tracks downloaded from the same GEO series for each dataset using p < 0.05, and annotated using ChIPseeker (v1.12.1).

**Echocardiography.** For echocardiography measurements, mice were anesthetized (2% isoflurane, 98% oxygen) and body temperature maintained at 37 °C. Echocardiography was performed using a 15-MHz linear ultrasound transducer (Vivid7; GE). LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD) mm mode measurements were made from short-axis views at the level of the papillary muscle. LVEDD was measured at the time of the apparent maximal LV diastolic dimension, whereas LVESD was measured at the time of the most systolic excursion of the posterior wall. LV fractional shortening (FS) was calculated as follows: FS = (LVEDD – LVESD)/LVEDD × 100%.

**Glyceral gradient column.** Nuclear extract harvested from HEK293T cells transiently transfected with VC-RREB1 (according to protocol described above) was layered on top of a continuous glycerol gradient (10–50%, or other factors (final concentration 50 mg/ml). EBV-suspension cells were pelleted to remove growth media, washed with PBS, and

**MAPPING assay.** Cells were plated into 12-well plate at 70–80% confluence. Cells were cultured in DMEM base media for 8 h and then stimulated with FBS (final concentration 1%), or other factors (final concentration 50 mg/ml). EBV-suspension cells were pelleted to remove growth media, washed with PBS, and
serum starved in DMEM base media. Following starvation, 5.0 × 10^6 cells were collected in Eppendorf tubes and stimulated with FBS. Cells were pelleted by centrifugation (280 × g for 10 min). All signaling reactions were carried out immediately after time points with 250 µl 2X sample buffer (100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 0.2% (v/v) bromophenol blue, 200 mM DTT). Reactions were analyzed by SDS-PAGE/western blot using standard protocols.

Mice. C57BL/6-J-REB1 mice were made at The Centre for Phenogenomics (The Hospital for Sick Children, Toronto, ON, Canada). All mice received environmental enrichment, animal rooms are maintained at 20–24 °C, 40–65% humidity, and 12 h light/dark cycle. Cas9 endonuclease-mediated cleavage was used in C57BL/6 J zygotes obtained from the Canadian Mouse Mutant Repository. Cas9 RNA guidanceed sites as follows (1): GCAGCTGCATACATGGCAAG, (2): GTC GCCTGCAAGGATACAC, (3): GAACTCGTTAATGGGACAG, (4): GTTTA CACAAAGGACCTT. All animal studies were approved by the Animal Research Council of the University Health Network (Toronto, Ontario, Canada). All experiments were performed on male mice.

Promoter assays. The promoter sequences were amplified by PCR with from human gDNA (Roche) and using Q5 High-fidelity 2x Master Mix (New England Biolabs). Full length PCR products were gel purified (Agarose 0.5% w/v), digested with Fermentas Fast digest reagents (Thermo Scientific) and cloned into the pGL3 Basic promoter (Promega) utilizing the Nhel/BglII sites. Ligation reactions were conducted with Rapid DNA ligation kit (Roche). The Dual-Luciferase Reporter Assay System (Promega) was used for promoter activation assays. Briefly, 2.0 × 10^5 cells were transfected with 100 ng of pGL3-promoter reporter construct and 4 ng of pRL-SV40 (Promega) using Lipofectamine-2000 (Invitrogen) according to the manufacturer’s protocol. 18 h post transfection, cells were lysed and assayed for firefly and renilla luciferase activity reading on a Glo-Max dual injector luminescer (Promega). Each measurement was made on 3 distinct samples repeated in triplicate. Error bars for luciferase activity represent standard deviations.

RAS activation assays. Serum starved cells were stimulated with 10% FBS (final concentration) for 1–12 min to activate RAS. Cells were washed with cold PBS, lysed with TX100 buffer (25 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton) supplemented with protease and phosphatase inhibitors (Roche) and incubated for 1 h at 4 °C with rotation. An aliquot was removed for analysis of total RAS in the whole cell lysate and the remainder incubated with 10µg GST-tagged Raf-binding domain (RBD, gift from Ikura lab). Complexes were immunoprecipitated with magnetic glutathione-agarose beads (Sigma Aldrich). IPs were washed with TX100 buffer and RAS-GTP eluted by boiling in 2X sample buffer.

RNA-seq analysis. Transcripts were quantified using STAR (v2.4.2a) and the UCSC genome browser (Feb 2009 assembly, hg38) as the reference and Gencode v25 for annotation. Differential analysis of quantified read counts from across the samples was facilitated by the DESeq2 (v1.16.1). Transcripts with zero reads mapped across all samples were filtered out prior to downstream analysis. Read counts were collapsed to gene level and transcripts with the highest reads mapped were kept. Lowly-expressed genes were filtered out by applying a minimum sum of at least 10 reads mapped in total per gene across the six samples. Fold changes were generated from the filtered count data matrix, modeled as a function of condition (CON vs. sh-RREB1), and p-values were further adjusted for multiple testing using a false discovery rate (FDR) of 1%. Significant hits were defined as genes with an FDR-adjusted p-value of at least 0.01 and an absolute log2-fold change greater than 1.

Statistical data analysis. Statistical analysis was performed using the R statistical environment (v3.4.1). Determination of significance for QPCR data was done using Student’s t-test, assuming equal variance, and p-values were calculated based on two-tailed test. Functional gene-annotation enrichment analysis, functional annotation, KEGG pathway mapping analysis of CHIP-seq and RNA-seq was FDR-adjusted and quantified with BIO-RAD Quantity One. All uncropped blots are provided in Supplementary Figs. 7 and 8.

X-ray imaging. X-rays were performed using the Faxitron UltraFocusDXA machine (Faxitron Biopics, LLC, Tucson, Arizona, USA) and the Automatic Exposure Control software. High-resolution X-ray images of skulls were acquired at approximately 25 kV for five seconds and 2x magnification.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. The RNA-seq and CHIP-seq data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE151417 for RNA-seq and GSE146902 for CHIP-seq. Source data are provided with this paper.

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Competing interests
The authors declare no competing interests.

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