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MicroRNA-15a and -16-1 act via MYB to elevate fetal hemoglobin expression in human trisomy 13

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Many human aneuploidy syndromes have unique phenotypic consequences, but in most instances it is unclear whether these phenotypes are attributable to alterations in the dosage of specific genes. In human trisomy 13, there is delayed switching and persistence of fetal hemoglobin (HbF) and elevation of embryonic hemoglobin in newborns. Using partial trisomy cases, we mapped this trait to chromosomal band 13q14; by examining the genes in this region, two microRNAs, mir-15a and -16-1, appear as top candidates for the elevated HbF levels. Indeed, increased expression of these microRNAs in primary human erythroid progenitor cells results in elevated fetal and embryonic hemoglobin gene expression. Moreover, we show that a direct target of these microRNAs, MYB, plays an important role in silencing the fetal and embryonic hemoglobin genes. Thus we demonstrate how the developmental regulation of a clinically important human trait can be better understood through the genetic and functional study of aneuploidy syndromes and suggest that mir-15a, -16-1, and MYB may be important therapeutic targets to increase HbF levels in patients with sickle cell disease and β-thalassemia.

erythropoiesis | globin gene regulation

H uman syndromes that are attributable to chromosomal imbalances or aneuploidy provide a unique opportunity to understand the phenotypic consequences of altered gene dosage (1, 2). Such observations also provide the prospect of gaining insight into the mechanisms mediating normal human development and physiology. However, in the vast majority of instances there is a limited understanding of how alterations in specific genetic loci contribute to the consequent phenotypic features seen in aneuploidy syndromes. Trisomy of chromosome 13 is one of the few viable human aneuploidies and is associated with a number of unique features (1), including a delayed switch from fetal to adult hemoglobin and persistently elevated levels of fetal hemoglobin (HbF) (1, 3). This trait is of considerable interest given that it is one of the few quantitative and objective biochemical phenotypes described in such syndromes. Additionally, the regulation of HbF is of great interest given the well-characterized role of elevated HbF in ameliorating clinical severity in sickle cell disease and β-thalassemia (6, 7).

During human development a series of switches occurs involving the transcription of the globin genes residing within the β-globin locus on human chromosome 11. A transient lineage of red blood cells, the primitive erythroid lineage, is produced in the first few weeks of human gestation (8). These cells produce a unique embryonic β-like globin chain, e-globin (9). Additionally, small amounts of other β-like globin genes are expressed in this lineage (8, 9). Subsequently, definitive erythrocyte cells are produced from long-term self-renewing hematopoietic stem cells. Initially these cells predominantly express the β-like fetal hemoglobin gene, γ-globin, and are produced in the fetal liver (10). Around the time of birth, when production of erythropoiesis and other hematopoietic cells shifts to the bone marrow, the predominant postnatal site for hematopoiesis, another switch occurs, resulting in down-regulation of γ-globin and concomitant up-regulation of the adult β-globin gene (6, 8, 10). There is a limited understanding of the molecular control of these globin gene switches that occur in human ontogeny, particularly with regard to the fetal-to-adult hemoglobin switch within the definitive erythroid lineage. Recent insight into these mechanisms has come from the field of human genetics (6) and resulted in the identification of the transcription factor BCL11A as a major regulator of this process (7). However, it is apparent that this factor cannot solely be responsible for this switch in ontogeny.

Results

Mapping of partial trisomy cases provides an opportunity to deduce genotype–phenotype relationships (1, 11). Given the dramatic reduction in births with trisomy 13 following the availability of prenatal diagnosis (12), mapping of such traits must rely on cases that have previously been cytogenetically mapped. Analysis of partial trisomy 13 cases has suggested that specific regions on the proximal part of chromosome 13 may be associated with elevations in HbF (1). Using eight well-annotated cases with detailed cytogenetic mapping data available (11), chromosomal band 13q14 appears to be unambiguously associated with elevated HbF levels (Fig. L4). By accounting for all 57 partial trisomy cases that have been reported with HbF measurements (SI Appendix, Fig. S1) (13, 14), with varying degrees of detail reported for cytogenetic mapping, a clear association with 13q14 is again deduced (Fig. 1B). This finding is strongly supported by Bayesian chromosomal region association models we developed (SI Appendix).

We then used an integrative genomic approach to identify candidates within the region implicated from the partial trisomy cases, by analyzing a gene expression compendium to search for genes with preferential expression in erythroid precursors (CD71+ relative to other cell types (SI Appendix) (15). Of the 76 genes in the region, 14 (18%) passed this test (SI Appendix, Fig. S2 and Table S1). We could further filter potential candidates by examining whether the histone 3 lysine 4 trimethylation (H3K4me3) modification, a well-characterized marker of active


The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE25678).

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transcription, was present in the proximal promoter of the genes from chromosomal band 13q14 in erythroid cell lines (defined as having a peak of H3K4me3 within 1.5 kb upstream from the transcription start site). Using data derived from the ENCODE project (16), we were able to find a number of unique peaks (SI Appendix, Table S2) and of the gene list established from the expression data, we could focus on 9 candidate genes (SI Appendix, Table S3). Of these, we noted that a top candidate in this region was a precursor RNA (DLEU2) for two microRNAs, 15a and 16-1, which have identical seed targeting sequences (Fig. 1C). This was of particular interest, given the role that microRNAs play in modulating various aspects of hematopoiesis and erythropoiesis specifically (17, 18). MiR-15a and -16 are expressed throughout human erythroid maturation in adult bone marrow cells and increase modestly with terminal differentiation (Fig. 1D and E), consistent with prior observations in cord blood and erythroid cell lines (19, 20).

These observations suggest that increased expression of miR-15a/16-1 in trisomy 13 could potentially result in elevated levels of HbF expression. To directly test this hypothesis, we used a lentiviral vector to increase expression of these microRNAs in adult bone marrow-derived hematopoietic progenitors that sub-

![Image](https://www.pnas.org/content/108/6/1520/suppl/DC1/)
gene expression were robustly increased by an average of 1.5-fold (SI Appendix, Fig. S3), similar to what would be expected in the context of a trisomy, we found that the levels of γ-globin gene expression were robustly increased by an average of 2.4-fold (Fig. 2A). Because trisomy 13 can result in elevated γ-globin synthesis even in newborns with elevated HbF levels at baseline (21), we increased expression of these microRNAs by 2- to 3-fold in human erythroleukemia K562 cells (SI Appendix, Fig. S4), which endogenously express high levels of γ-globin, and found that the γ-globin levels could be further increased by 50% (Fig. 2B). Newborns with trisomy 13 also show elevated expression of the embryonically expressed ε-globin, as illustrated by the persistence of low level hemoglobin Gower 2 (ε2G) expression (4). This increase was recapitulated in the primary bone marrow-derived cells with increased miR-15a/16-1 expression (Fig. 2C). Since alterations in γ-globin expression may accompany altered differentiation of cells, we examined the lentivirally transduced primary adult erythroid progenitors and found no major differences in the morphology or phenotype of cells with increased miR-15a/16-1 expression compared with controls (Fig. 2D and SI Appendix, Fig. S5). To gain further insight into the mechanism by which these microRNAs may be acting to elevate γ-globin expression, we assessed cell cycle progression in the synchronously differentiating primary erythroid cells (7). Interestingly, we noted that cell cycle progression was slowed by miR-15a/16-1 overexpression at the early stages of differentiation that represent colony-forming unit erythroid cells (CFU-Es) or pro-erythroblasts (G1- and S-phase difference, P < 0.001), but was equivalent to controls at the later stages that represent more mature (basophilic) erythroblasts (Fig. 2E and F) (7). Such findings are reminiscent of the observations made using S-phase inhibitors for HbF induction in primate models, where the most responsive stages appeared to be the CFU-Es and proerythroblasts (22, 23).

To gain further insight into the molecular etiology for these observations, we examined whether specific miR-15a/16-1 targets may be potential mediators of the increased HbF expression. Because evolutionary conservation of seed-matched targets shows great utility in identifying bona fide microRNA targets (24), we used a metric of target context and conservation (aggregate $P_{CT}$, which is a Bayesian estimate of the probability that a site is conserved due to selective maintenance of miRNA targeting rather than by chance) and compared this with relative expression of miRNAs in the erythroid target tissues of interest relative to a large number of other tissues (Fig. 3A and SI Appendix, Fig. S6). Using this approach, MYB was one miR-15a/16-1 target that appeared to be of great interest, given that it was highly expressed in erythroid progenitors and had two conserved 8mer miR-15a/16-1 targeting sites (Fig. 3B). This was particularly notable, because common genetic variants from genomewide association studies have suggested that polymorphisms in the MYB locus are important mediators of variation in HbF levels in humans (25), which is further supported by the finding that overexpression of MYB in cell lines causes a decrease in γ-globin expression (26). We found that MYB protein levels were reduced with even a modest (two- to threefold) increase in miR-15a/16-1 expression in erythroid cell lines (Fig. 3C) and a luciferase reporter assay confirmed that

![Graphs and Diagrams](Fig. 2) **Fig. 2.** Increased expression of miR-15a/16-1 in human erythroid cells results in elevated HbF and embryonic globin gene expression. (A) Percentage of γ-globin gene expression (as a percentage of all human β-like globin genes) in cells transduced with pLVX-puro control or pLVX-miR-15a/16-1 lentivirus (n = 3 per group; ***P < 0.001). Measurement was at the basophilic erythroblast stage of differentiation (days 6–7 of differentiation). (B) Relative amount of γ-globin gene expression in K562 cells transduced with pShP6PUW control or miR-15a/16-1 containing lentivirus, following selection (n = 3 per group; *P < 0.02). (C) Percentage of γ-globin gene expression (as a percentage of all human β-like globin genes) in primary bone marrow CD34-derived cells transduced with pLVX-puro control or pLVX/miR-15a/16-1 lentivirus (n = 3 per group; **P < 0.01). (D) Representative cytospin images of primary bone marrow CD34-derived cells transduced with pLVX-puro control or pLVX/miR-15a/16-1 lentivirus (taken with a 63× objective lens). All cells show similar size and morphological distribution on days 5–6 of differentiation. At other stages of differentiation the control and miR-15a/16-1 transduced cells also had the same morphology. (E and F) Cell cycle analysis of primary bone marrow CD34+-derived cells transduced with pLVX-puro control or pLVX/miR-15a/16-1 lentivirus on day 4 (E) and day 7 (F) of differentiation (n = 3–4 per group). All data are shown as the mean ± SD.
the MYB 3'-UTR is a direct target of these microRNAs (Fig. 3D), consistent with previous studies (20, 27, 28).

To test whether MYB may be a critical mediator of γ-globin expression, we reduced MYB expression with two shRNAs in synchronously differentiating adult erythroid progenitors (Fig. 3E). This knockdown robustly increased γ-globin expression, as occurs to a lesser extent with miR-15a/16-1 elevation (Figs. 2D and 3F). Concomitantly, we found that expression of the embryonic globin chain, ε-globin, was also dramatically increased by MYB knockdown (Fig. 3G). Our examination of expression data from a recent study of MYB siRNA treatment of umbilical cord blood erythroid progenitors (29) demonstrated robust elevations

Fig. 3. MYB is a target of miR-15a/16-1 and regulates HbF expression. (A) By comparing the aggregate PCT of a variety of miR-15 or -16 seed targets (24) with the log2 normalized relative expression in early (CD34+ hematopoietic/erythroid progenitors (relative to a panel of 78 other human cells and tissues), MYB appears to be a standout candidate target (highlighted in red with arrow). The x-axis plots aggregate PCT (24) on a linear scale, whereas the y-axis shows relative expression in the erythroid progenitors as a log2 ratio. (B) Two 8mer target sites for miR-15a and -16-1 are located in the 3'-UTR of MYB. (C) Ectopic expression of miR-15a/16-1 in K562 cells at a level two- to threefold of normal results in reduction in MYB protein levels; GADPH was used as a loading control for this Western blot. (D) Cotransfection of 293T cells with control or miR-15a/16-1 expression vector (in pLVX) and a MYB 3'-UTR construct luciferase reporter (in psiCHECK-2 vector) show reduced luciferase activity with elevated microRNA expression. (E) Relative MYB expression is shown on day 5 of differentiation in primary human CD34+-derived cells transduced with plKO.1 control or plKO.1 with shRNAs targeting MYB (n = 3 per group; ***P < 0.001). (F and G) Percentage of γ-globin (F) and ε-globin (G) gene expression as a percentage of all human β-like globin genes on day 7 of differentiation in cells transduced with plKO.1 control or plKO.1 vector expressing two different shRNAs targeting MYB (n = 3 per group; ***P < 0.001). (H) Gene set enrichment analysis (30) of a monocyte gene expression signature, composed of 371 genes (31), evaluated using the expression array data of shMYB cells versus controls (n = 4 per group), demonstrates global up-regulation of the monocyte gene signature in the shMYB cells. Enrichment plot is shown above heat map (below) using a green line to show the running enrichment score. (I) Representative flow cytometry profiles of propidium iodide staining in K562 cells transduced with empty or shMYB containing plKO.1 lentiviruses. Data are shown as an average of three experiments for each group (P < 0.001 for both shMYB experiments relative to controls). All data are shown as the mean ± SD.
of both \( \gamma \)-globin and \( \epsilon \)-globin gene expression, even with higher baseline levels of these globin genes at this stage of human ontogeny (SI Appendix, Fig. S7).

Examination of cells with a knockdown of MYB demonstrated an increased presence of myeloid (primarily monocyte) cells and signs of precocious erythroid differentiation by morphological examination (SI Appendix, Fig. S8). To more precisely define the molecular basis of these changes, expression analysis was performed with cells from one of the MYB knockdowns along with a set of controls. We did not observe any significant difference in mRNA expression levels of known regulators of erythropoiesis and globin gene expression, including \( BCL11A, GATA1, KLF1 \) (EKLF), \( ZFP1M1 \) (FOG-1), and \( SOX6 \), that we and others have previously described (7, 10) (SI Appendix, Fig. S9). Gene set enrichment analysis (30) was used with gene sets derived from lineage-specific and differentiation stage-specific expression datasets (SI Appendix). A marked increase in the expression of the monocyte gene set was notable in the shRNA-treated cells compared with controls (Fig. S9) (31). In permissive conditions, a similar type of knockdown can also result in increased production of megakaryocytes (19). Moreover, when gene sets were created from the MYB knockdown expression sets compared with controls, the up-regulated genes were found to be significantly enriched in the later stages of erythroid differentiation, supporting the notion that precocious erythroid differentiation was occurring in this context (SI Appendix, Fig. S10) (32). Together, these findings suggest that MYB is necessary for the normal differentiation kinetics of adult erythroid cells and reduction in the level of this gene results in altered erythroid differentiation kinetics and the increased presence of cells from other lineages. Consistent with the findings with moderate MYB knockdown by miR-15a/16-1, these shRNAs were able to result in a marked slowing of cell cycle progression (Fig. 3I) and marked up-regulation of \( \gamma \)-globin expression in erythroid cell lines (SI Appendix, Fig. S11). In support of the slowing of cell cycle progression, we noted that the expression of several cell cycle regulators were altered in our expression data from the MYB knockdown cells compared with controls (SI Appendix, Fig. S12). The altered cell cycle and differentiation kinetics of cells with MYB knockdown are consistent with findings in erythroid cells using hypomorphic \( myb \) alleles in mice (33). Our findings suggest that MYB plays a critical role coordinating globin gene expression, cell cycle regulation, and erythroid differentiation.

Alterations in \( \gamma \)-globin expression can occur in the context of stress erythropoiesis or other states where erythroid differentiation is perturbed. Patients with trisomy 13 and elevated HbF levels do not have anemia (4, 21), but the evaluation of in vivo erythropoiesis in these patients has not previously been reported. To properly ascertain this, we examined autopsy specimens from patients with trisomy 13 from the archives ranging over four decades at a single institution (SI Appendix). Initially all available trisomy 13 cases were selected for evaluation, of which 17 were used for our final analysis on the basis of confirmation of the diagnosis of trisomy 13 and presence of appropriate histological samples (SI Appendix, Table S4). In all of the samples examined, erythropoiesis appeared appropriate for age, and full maturation of the erythroid lineage could be appreciated (Fig. 4 A, C, and D). This suggests that the elevated HbF levels in trisomy 13 can occur without grossly perturbed erythropoiesis. In examining other aspects of hematopoiesis, we found a dramatic increase in megakaryocyte numbers in over 70% of the cases examined (Fig. 4 B and C and SI Appendix, Table S4). In addition, the majority of the cases (64%) showed abnormal nuclear morphology of the megakaryocytes, suggestive of decreased ploidy in these cells (Fig. 4 B–D and SI Appendix, Fig. S13). These findings were specific to patients with trisomy 13, as autopsy specimens from the same institution, in the same time period, and in patients of similar ages with other diagnoses lack these findings (SI Appendix). Interestingly, MYB knockdown in human cells and hypomorphic alleles in mice show similar phenotypes in megakaryocytes (19, 34–36), suggesting that miR-15a/16-1 overexpression and the consequent reduction in MYB expression may alter other aspects of hematopoiesis in trisomy 13 patients.

Discussion

We have demonstrated, using a combination of genetic and functional approaches in human cells that the overexpression of miR-15a/16-1 results in elevations in HbF gene expression and this likely explains why patients with a trisomy of chromosome 13 have a delayed fetal-to-adult hemoglobin switch and persistence of fetal hemoglobin. This effect is mediated, at least in part, through down-modulation of the MYB transcription factor, which we have shown is a potent negative regulator of HbF expression and is a direct target of miR-15a and -16-1 (Fig. 5). It is possible that other genes on chromosome 13 may also contribute to this phenotype, but miR-15a and -16-1 appear to have a major role in its regulation.
impact. Our study demonstrates a unique and previously unappreciated pathway that regulates this intensively studied developmental process. The exact relevance of our findings to normal physiology is not clear, but it appears to be likely that these pathways may play an important role in the normal fetal-to-adult hemoglobin switch (Fig. 5). Consistent with this notion, common genetic variants close to the MYB gene appear to be important regulators of HbF levels in adult humans (25). Further work will be needed to gain insight into the exact role of these factors in the mechanisms mediating human hemoglobin switching. Nonetheless, it is apparent from our work that miR-15a, -16-1, and MYB could be important therapeutic targets to elevate HbF expression to ameliorate the severity of sickle cell disease and β-thalassemia.

Our findings demonstrate the power of using human genetic approaches to understand developmental processes, where the use of model organisms can be limited (37). Importantly, our findings suggest that alterations of gene dosage at specific genetic loci likely underlie the numerous phenotypes observed uniquely with specific aneuploidy syndromes (1). In relatively few cases has this been strongly supported by functional evidence. Similar types of approaches may help to uncover other genotype-phenotype correlations in these fascinating syndromes that can teach us a great deal about normal human development and physiology.

Materials and Methods

Details of the cell culture approaches, constructs used, lentiviral preparation, RNA analysis, flow cytometry, protein methods, and analysis can be found in SI Appendix. CD34+ cells were cultured and differentiated using a two-phase culture method in serum-free conditions, with appropriate cytokines added at the various stages of the culture (7). RNA extraction, quantitative RT-PCR, and microarray expression analysis were performed in a manner similar to what has previously been described (7, 38). Lentivirus production and infection was carried out using a modified spin-infection method for the erythroid progenitors that were grown in suspension (39). Flow cytometry was performed using standard approaches (38) with data analysis occurring in the FlowJo 7.5.5 software suite.

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