SUPPLEMENTAL METHODS

DS-ALL case-control studies

Children's Oncology Group (COG)/National Down Syndrome Project (NDSP) study 1 - Cases consisted of 312 individuals with DS treated on COG ALL trials for recently diagnosed ALL (2000-2013) with existing genotype data. Germline DNA was extracted from peripheral blood samples collected at clinical remission once circulating blasts were eliminated. Controls included a total of 501 unrelated individuals with trisomy 21, confirmed by karyotype, without a known history of ALL enrolled in the NDSP, with germline DNA isolated from lymphoblastoid cell lines (LCLs) using Puragene DNA purification kit (Qiagen, Valencia, CA) as part of a multicenter GWAS of congenital heart defects in DS.[1] Both cases and controls were genotyped on the Affymetrix Single Nucleotide Polymorphisms (SNP) 6.0 array (Affymetrix, Inc, Santa Clara, CA) in accordance with manufacturer instructions. Genotype calling was conducted in Affymetrix Genotyping Console Software, version 4.2, using the Birdseed v2 algorithm.

COG/NDSP study 2 - An additional 134 cases of DS-ALL were identified from ALL cases diagnosed 2011-2015 and treated on COG ALL trials. Similarly, a second independent set of 358 DS controls without a known ALL diagnosis were included from the NDSP.[1] Remission germline DNA samples from DS-ALL cases were genotyped on the Infinium Omni2.5Exome array (Illumina, Inc, San Diego, CA), while DS control germline samples were genotyped separately on the Illumina HumanOmni1 array. Genotype calls were generated in Illumina GenomeStudio Genotyping Module v2.0.

Michigan-based DS-ALL (MiDSALL) study - Neonatal dried bloodspots (DBS) were obtained from the Michigan Neonatal Biobank for 25 DS non-ALL controls and 398 DS-ALL cases, identified through linkage between the Michigan Department of Health and Human Services

birth records and the Michigan Cancer Surveillance Program. DNA was extracted from neonatal (DBS) using the GenSolve DNA extraction kit (GenTegra, Pleasanton, CA). Extracted DNA was genotyped on Illumina's Infinium Global Screening Array and genotype calls generated for SNPs using Illumina GenomeStudio Genotyping Module v2.0 software.

International Study of Down Syndrome Acute Leukemia (IS-DSAL) - The IS-DSAL consisted of 187 DS-ALL cases and 200 DS non-ALL controls. Of the 187 cases, 109 were identified from 3,234 ALL cases with unknown trisomy 21 status from the California Cancer Records Linkage Project (CCRLP) ALL GWAS (Principal investigators, PIs: Joseph Wiemels and Xiaomei Ma),[2] which includes cases of childhood ALL (diagnosed 0-14 years of age) born in California between 1982 and 2009 with records available in the California Cancer Registry (year of diagnosis 1988-2011). DNA samples were obtained for additional DS-ALL cases from several Childhood Leukemia International Consortium (CLIC) studies, including the California Childhood Leukemia Study (*n*=28, PI: Catherine Metayer),[3] the Washington State ALL study (*n*=19, PI: Beth Mueller),[4] the UK Childhood Cancer Study and University of Manchester (*n*=9, PI: Jillian Birch),[5] a Brazilian ALL study (*n*=9, PI: Maria Pombo-de-Oliveira),[6] and the Quebec childhood ALL cohort (*n*=3, PI: Daniel Sinnett).[7] Additional DS-ALL cases (*n*=10) were received from the Children's Hospital of Michigan (*n*=10, PI: Jeffrey Taub). Neonatal DBS were obtained from DS controls, without an ALL or acute myeloid leukemia diagnosis by 15 years of age, from the California Biobank Program, and through linkage between the California Department of Public Health Genetic Disease Screening Program and the California Cancer Registry. DNA was extracted from neonatal DBS using the Qiagen DNA Investigator blood card protocol. Cases and controls were genotyped on the Affymetrix Axiom World LAT Array,[2] and the array data were processed with Affymetrix Power Tools software to generate genotype calls for all variants.

Confirmation of Trisomy 21

To confirm trisomy 21 in samples obtained from individuals with uncertain or unknown DS, the mean genotype log R ratio (i.e., hybridization intensity) of SNPs on chromosome 21 was calculated for each sample. Twenty-five, randomly selected positive control samples with known trisomy 21, confirmed by karyotype, were included from the NDSP. Samples with a mean log R ratio for chromosome 21 >3 standard deviations from the mean across samples were assigned as trisomy 21.

Genotype Quality Control

We excluded samples with call rate <95% or gender discrepancies. We calculated pairwise identity-by-descent estimates for all individuals included in the study populations, and removed one member of each pair with PI-HAT >0.20. We removed non-autosomal SNPs, autosomal SNPs with call rates <95%, SNPs with significant differential missingness between cases and controls within a population (P<0.01), and SNPs with significant departure from Hardy-Weinberg equilibrium (HWE) using controls in each study population (P<1.0×10^{-4}).

Genetic Ancestry Analysis

In each study, directly genotyped SNP data were merged with HapMap Phase 3 samples of Northern and Western European (CEU), East Asian (CHB), West African (YRI), and Mexican (MEX) ancestry.[8] In the merged dataset, genetic ancestry was estimated using STRUCTURE v.2.3.4 software on a set of ancestry informative markers overlapping with those reported by Yu *et al*.[9] Ancestral proportions were estimated assuming four underlying subpopulations, and individuals were assigned to the following groups: European (≥80% CEU ancestry), African (≥50% YRI ancestry), Asian (≥85% CHB ancestry), or Hispanic (≥15% MEX ancestry and proportion MEX ancestry greater than proportion YRI ancestry). Individuals not meeting any of the ancestry group definitions were considered admixed. In each of the four

studies, association analyses were restricted to population ancestry groups with at least 15 cases and 15 controls. These consisted of: 1) Europeans (226 cases, 374 controls) and Africans (15 cases, 62 controls) in COG/NDSP study 1; 2) Europeans (78 cases, 292 controls) and Hispanics (46 cases, 44 controls) in COG/NDSP study 2; 3) Europeans (20 cases, 275 controls) in MiDSALL; and 4) Europeans (62 cases, 53 controls) and Hispanics (95 cases, 92 controls) in IS-DSAL.

Chromosome 21 Genotype Calling and Analysis

To account for ploidy effects on genotype calling in samples with trisomy 21, we applied a K-means unsupervised clustering algorithm in R as previously described for calling trisomy 21 genotypes.[1, 10] We limited chromosome-wide analysis to subjects genotyped on Affymetrix arrays (*i.e.* COG/NDSP study 1 and IS-DSAL), for which the algorithm was designed. Chromosome 21 variants were analyzed using logistic regression, assuming additive allelic effects with 0, 1, 2, or 3 possible alternate allele copies. Association analyses were stratified on study and genetic ancestry group. Manual inspection of genotype clusters was carried out for SNPs with significant associations ($P<5.0\text{x}10^{-8}$), and replication attempted by visually inspecting and manually calling variants if present on-array in one or more of the studies genotyped on Illumina platforms (*i.e.* COG/NDSP study 2 and MiDSALL).

Analysis of ALL GWAS SNPs in DS versus non-DS individuals

We assessed the risk effects of seven well-replicated childhood ALL GWAS SNPs, in *IKZF1*, *CDKN2A*, *ARID5B*, *CEBPE*, *GATA3*, *BMI1*, and *PIP4K2A*, in DS individuals versus non-DS individuals. Initial analyses were conducted using genotype data from the non-DS California Cancer Records Linkage Project (CCRLP) ALL GWAS,[2] including 3,082 non-DS ALL cases (1,928 Hispanic, 1,154 European) and 3,306 non-DS controls (2,092 Hispanic, 1,214 European). Logistic regression models assuming additive allelic effects were generated for

case-case (DS-ALL vs. non-DS ALL) and control-control (DS vs. non-DS control) comparisons for each SNP, adjusting for sex. Ethnicity-stratified results were combined using fixed-effects meta-analysis, using Cochran's *Q* test to test for heterogeneity between Hispanic and European studies, using *P* cutoff 0.1.

We next assessed whether molecular subgroup, as defined below, influenced differences in risk allele frequency at the seven ALL GWAS SNPs between DS-ALL and non-DS ALL cases. Analyses were limited to 255 DS-ALL cases in the COG/NDSP studies and 2,387 non-DS B-ALL cases from COG9900 or AALL0232 trials for which data were available for common B-ALL molecular subgroups high hyperdiploidy and ETV6-RUNX1 fusion, and for CRLF2 expression status. Cases were assigned to one of four subgroups: CRLF2-high (151 DS-ALL; 93 non-DS ALL), high hyperdiploidy (19 DS-ALL; 888 non-DS ALL), *ETV6-RUNX1* fusion (45 DS-ALL; 547 non-DS ALL), and "B-other" (40 DS-ALL; 859 non-DS ALL). High hyperdiploidy was defined as ≥51 chromosomes in non-DS ALL and ≥52 chromosomes in DS-ALL cases. Cases were classified as B-other if they were negative for CRLF2-high, high hyperdiploidy, *ETV6-RUNX1*, *E2A-PBX1*, *KMT2A* rearrangement, or t(8;14)(q11;q32) (the latter in DS-ALL only). We excluded two DS-ALL and 5 non-DS cases with concurrent CRLF2 overexpression and high hyperdiploidy. PCs were calculated from directly genotyped SNPs and retained for downstream statistical analyses to adjust for genetic ancestry. Separate logistic regression models were constructed to compare frequency of SNP risk alleles between DS-ALL and non-DS ALL cases: 1) overall, adjusting for the top 5 PCs; 2) overall, adjusting for the top 5 PCs and molecular subgroups; and 3) within each molecular subgroup, adjusting for the top 5 PCs. For each SNP, ORs were calculated for the association of each additional risk allele with DS status. For comparison with effect sizes of the same SNPs in non-DS ALL, logistic regression tests were carried out comparing allele frequencies between COG non-DS ALL cases and controls from the Multi-Ethnic Study of Atherosclerosis (MESA; dbGaP Accession: phs000209.v10.p2),[11] both overall and stratified by molecular subgroup.

Luciferase reporter assays

The gDNA sequences from CEU LCLs homozygous for either the non-risk (GM12878) or risk allele (GM12874) of rs58923657 were used as templates to synthesize (Genscript, Piscataway, NJ) the strong candidate enhancer overlapping rs6964969 and rs58923657 on chr7 from 50472801-50473400 (hg19). The non-risk and risk variant enhancers were ligated into the pGL3-Promoter vector (Promega, Madison, WI) using BamHI and XhoI. The allele-specific reporter vectors and an internal control vector expressing *Renilla* luciferase (pRL-SV40; Promega) were co-transfected with into DS and non-DS LCLs using a Neon Transfection System (Thermo). Luciferase activity was measured in lysates after 24 hours using a Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity of each sample was measured by taking the ratio of firefly/*Renilla* luminescence and normalizing to the empty pGL3-Promoter vector. We performed reporter experiments three times, measured luciferase activity in 3 replicate transfected wells, and tested the difference in luciferase activity was assessed by Student's 2-tailed t test.

Electrophoretic mobility shift assays

Nuclear protein was extracted from DS and non-DS LCLs using a nuclear extraction kit (Abcam, Cambridge, UK). Complimentary DNA oligos (Integrated DNA Technologies, Coralville, IA) matching the sequences encompassing SNPs rs62445866, rs6964969, rs6944602, rs10264390, and rs17133807 (**supplemental Table 5**) were annealed to generate unlabeled and 5'-IRDye 700-labeled EMSA probes. DNA binding reactions contained 50 ng of labeled probed, 1X binding buffer (10 mM Tris, pH 7.5, 30 mM KCl, 2 mM DTT, 0.5 mM EDTA), 50 ng poly-dI.dC, and 1 µg nuclear extract. Binding reactions were incubated for 30 minutes in the dark at room temperature. Binding specificity was assessed by including 10-fold molar excess of unlabeled probe with the same sequence. Samples were mixed with nucleic acid loading buffer

and resolved on non-denaturing 5% TBE gels (Bio-Rad, Hercules, CA) at 4°C and imaged with a LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Lentivirus generation and transduction for lymphoid proliferation assay

HEK-293T cells were transfected with a lentiviral vector encoding either IKZF1 or control non-targeting (NT)-shRNA (MISSION® pLKO.1-IKZF1-shRNA-puro-GFP and pLKO.1-NT-puro-GFP, MilliporeSigma, St. Louis, MO) using Lipofectamine 3000 (ThermoFisher). LCLs were transduced with virus supernatants and selected in 0.5–2 ug/mL puromycin for 9–12 days. GFP+ cells were sorted using a SH800S Cell sorter (Sony Biotechnology, San Jose, CA). IKZF1 knockdown was confirmed by Western blot using rabbit anti-IKAROS (Cell Signaling Technology, clone 5443, Danvers, MA) and mouse anti-beta actin primary antibodies (Cell Signaling Technology, clone 8H10D10), followed by goat secondary antibodies (LI-COR). Blots were imaged using a LI-COR Odyssey Infrared Imaging System.

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1 – Enhancer reporter assays for rs58923657 in patient-derived (A) DS (n=10) and (B) non-DS LCLs (n=10). The relative luciferase activity from cell lysates was measured 24h later using a Dual Luciferase Reporter assay (Promega). Data shown are mean ± standard error of the mean (SEM) from transfections performed in triplicate and the difference in luciferase activity was assessed by Student 2-tailed t test.

Figure S2 – Protein-DNA interactions at the indicated SNPs were analyzed using by EMSA. Nuclear extracts from DS (n=3) and non-DS (n=3) LCLs were incubated with 5'-IRD700-labeled allele-specific probes containing genomic sequence flanking the indicated SNP. Excess unlabeled competitor DNA was used as a control to demonstrate specific binding.

Figure S3 – IKAROS protein levels in LCLs expressing IKZF1-shRNA (+) compared to non-targeting (NT) shRNA (-), by Western blot analysis.