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# Leukocyte adhesion deficiency-III is caused by mutations in *KINDLIN3* affecting integrin activation

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**Integrins are the major adhesion receptors of leukocytes and platelets. $\beta_1$ and $\beta_2$ integrin function on leukocytes is crucial for a successful immune response and the platelet integrin $\alpha_{IIb}\beta_3$ initiates the process of blood clotting through binding fibrinogen[1–3]. Integrins on circulating cells bind poorly to their ligands but become active after 'inside-out' signaling through other membrane receptors[4,5]. Subjects with leukocyte adhesion deficiency-1 (LAD-I) do not express $\beta_2$ integrins because of mutations in the gene specifying the $\beta_2$ subunit, and they suffer recurrent bacterial infections[6,7]. Mutations affecting $\alpha_{IIb}\beta_3$ integrin cause the bleeding disorder termed Glanzmann's thrombasthenia[3]. Subjects with LAD-III show symptoms of both LAD-I and Glanzmann's thrombasthenia. Their hematopoietically-derived cells express $\beta_1$, $\beta_2$ and $\beta_3$ integrins, but defective inside-out signaling causes immune deficiency and bleeding problems[8]. The LAD-III lesion has been attributed to a C→A mutation in the gene encoding calcium and diacylglycerol guanine nucleotide exchange factor (*CALDAGGEF1*; official symbol *RASGRP2*) specifying the CALDAG-GEF1 protein[9], but we show that this change is not responsible for the LAD-III disorder. Instead, we identify mutations in the *KINDLIN3* (official symbol *FERMT3*) gene specifying the KINDLIN-3 protein as the cause of LAD-III in Maltese and Turkish subjects. Two independent mutations result in decreased *KINDLIN3* messenger RNA levels and loss of protein expression. Notably, transfection of the subjects' lymphocytes with *KINDLIN3* complementary DNA but not *CALDAGGEF1* cDNA reverses the LAD-III defect, restoring integrin-mediated adhesion and migration.**

Individuals with LAD-III (also called LAD-I variant)[10] have Glanzmann's thrombasthenia–like bleeding problems and LAD-I–like life-threatening infections. Their leukocytes fail to undergo $\beta_2$ and $\beta_1$ integrin–mediated adhesion and migration despite normal integrin expression, which is characteristic of the LAD-III disorder. We have investigated the nature of LAD-III lesions in a Maltese subject (family one)[11] and two Turkish subjects, one previously described (family two; family five in ref. 12) and the other characterized here for the first time (family three; details in the Methods and **Supplementary Fig. 1** online). Given the likelihood that LAD-III in the Turkish and Maltese families is a recessive condition caused by mutations inherited from a common ancestor, we used homozygosity mapping to show the most likely location of the gene responsible for the LAD-III disorder to be a region between 60.6 megabases (Mb) and 65.3 Mb on chromosome 11 (**Supplementary Fig. 2** online).

The *CALDAGGEF1* gene (chromosome 11q13.1: 64.25–64.27 Mb) lies on the distal boundary of this region. CALDAG-GEF1 is activated through binding of diacylglycerol and $Ca^{2+}$ and is a guanine exchange factor for Rap1, a GTPase that has an essential role in the activation of integrins (refs. 13,14). The gene encodes two proteins as a result of alternative splicing, a 68-kDa cytosolic form and a 72-kDa form that is membrane localized owing to an additional amino-terminal myristoylated and palmitoylated domain[15]. Caldag-gef1–deficient mice mimic the LAD-III phenotype, showing Glanzmann's thrombasthenia–like bleeding problems[16] and defective function of neutrophil $\beta_1$ and $\beta_2$ integrins[17]. A C→A base change in the *CALDAGGEF1* gene has been described recently in two Turkish subjects with LAD-III[9]. This mutation, in a putative splice acceptor site for exon 16, is reported to cause a splicing problem leading to a loss of *CALDAGGEF1* mRNA and protein expression and is suggested to be responsible for LAD-III.

Sequencing of all 18 exons and intron-exon boundaries of the *CALDAGGEF1* gene for the three subjects with LAD-III and their relatives revealed that the Turkish subjects with LAD-III were homozygous for the C→A base change (**Fig. 1a** and **Supplementary Fig. 3** online). The parents of the Turkish subjects were heterozygous, whereas the sister in family two was homozygous for the normal C allele. In contrast, the C→A change was not present in the Maltese family, and no further nucleotide changes were detected in the *CALDAGGEF1* gene (data not shown). The observation that a C→A change was present in the two Turkish subjects with LAD-III in this study and also in the previously described two Turkish subjects[9], but not in the Maltese subject, suggests that this mutation is exclusive to LAD-III subjects of Turkish origin and implies a Turkish founder effect maintained through consanguineous marriage.

The C→A mutation has been reported to prevent correct splicing of the *CALDAGGEF1* transcript, resulting in unstable *CALDAGGEF1*

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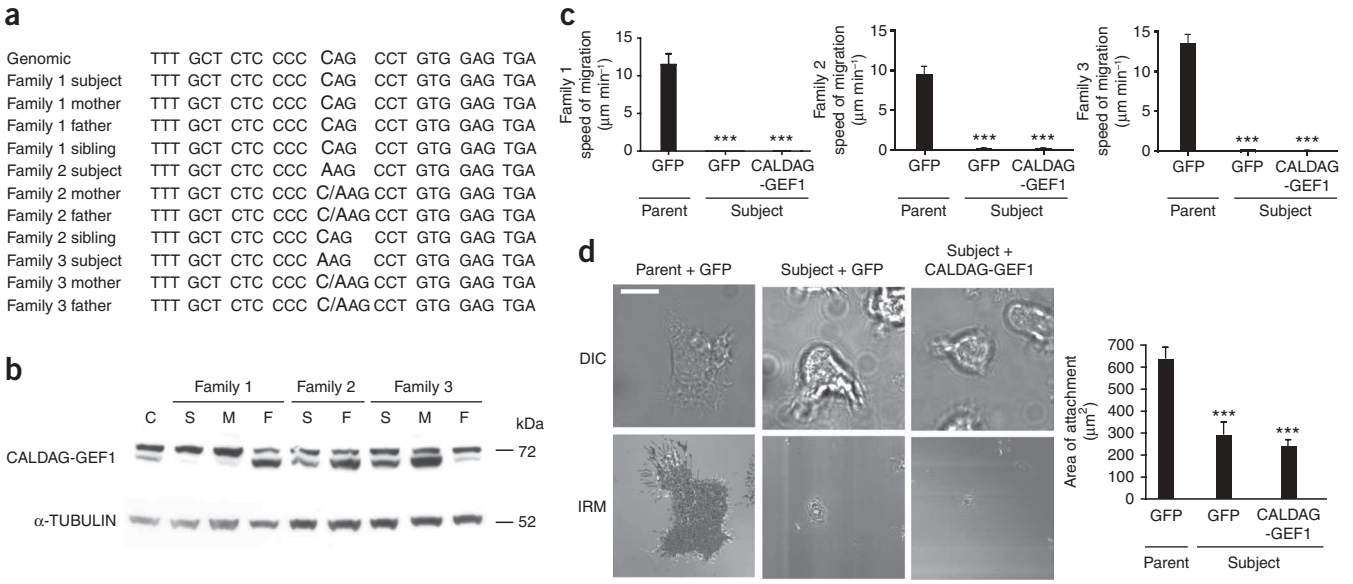


**Figure 1** The *CALDAGGEF1* gene C→A base change has no effect on mRNA and protein levels or on deficient LAD-III B cell adhesion and migration. (**a**) The DNA sequence surrounding the C→A base change in exon 16 of the *CALDAGGEF1* gene in the Turkish (C→A base change) and Maltese (no base change) families. C or A indicates homozygosity; C/A indicates heterozygosity. The original data are shown in **Supplementary Figure 1.** (**b**) CALDAG-GEF1 protein abundance in subjects (S), relatives (M, mother; F, father) and control individuals (C), as assessed by western blotting ($n = 2$ independent samples per family). α-TUBULIN was used as a loading control. (**c**) Migration characteristics of LAD-III B cells adhered to ICAM-1. LAD-III cells from each family were transfected with a wild-type *CALDAGGEF1* or EGFP cDNA construct; EGFP–transfected parents' (families 1 and 3, mother; family 2, father) cells are shown for comparison. $n = 4$ independent experiments for each family. Data are shown as means ± s.e.m.; $^{***}P < 0.001$. (**d**) Left, differential interference contrast (DIC) and IRM images of B cells from the family 3 subject with LAD-III and from the mother (Parent) transfected as in **c** ($n = 2$). Scale bar, 10 μm. Right, quantification of the area of close contact for $n = 35$ cells per cell type. Data are shown as means ± s.e.m.; $^{***}P < 0.001$.

mRNA[9]. Quantitative RT-PCR with probes that recognize either total *CALDAGGEF1* mRNA (**Supplementary Fig. 4** online), or the larger alternatively spliced form of *CALDAGGEF1* mRNA (data not shown), revealed that the subjects with LAD-III all expressed *CALDAGGEF1* mRNA, although at somewhat variable levels. Furthermore, all subjects with LAD-III expressed the 72-kDa and 68-kDa CALDAG-GEF1 proteins (**Fig. 1b**). In summary, we find that the C→A change present in the Turkish subjects had no impact on either CALDAG-GEF1 mRNA or protein levels, in contrast to results previously described[9]. Finally, the C→A base change was not present in the Maltese family.

To test the possibility that the C→A base change might affect the function of CALDAG-GEF1, we generated Epstein-Barr virus (EBV)-transformed B cells from the subjects with LAD-III and their parents. The parents' B cells, but not the subjects' B cells, were able to attach and migrate without added stimulant on the intercellular adhesion molecule-1 (ICAM-1) that is the ligand of the $\beta_2$ integrin lymphocyte function associated antigen-1 (LFA-1; **Fig. 1c**). Migration on fibronectin, which is mediated by $\alpha_4\beta_1$ and $\alpha_5\beta_1$, showed the same difference (data not shown). Expression of a transfected human *CALDAGGEF1* cDNA construct that includes the membrane localization domain[15] had no effect on the migration of the B cells from subjects with LAD-III (**Fig. 1c**) or on their individual cell tracking patterns (data not shown). A similar negative result was obtained with cDNAs encoding either human or mouse cytosolic CALDAG-GEF1 (data not shown).

We next used interference reflection microscopy (IRM) to investigate more directly the effect of CALDAG-GEF1 on cell adhesion to ICAM-1. Cells from family 3 subject's mother showed a pattern of close contact with ICAM-1, whereas cells from the family 3 subject were only lightly attached, whether or not they expressed wild-type CALDAG-GEF1 (**Fig. 1d**). We reached the same conclusion after quantification of the areas of attachment (**Fig. 1d**). These experiments show that expression of several forms of wild-type CALDAG-GEF1 fails to repair the adhesion and migration defect caused by the LAD-III disorder.

Taken together, these data show that mutation in the *CALDAGGEF1* gene is not the cause of the LAD-III disorder in the subjects in our study. Moreover, the properties of CALDAG-GEF1 do not totally conform to the characteristics of LAD-III disorder, in which the functions of $\beta_1$, $\beta_2$ and $\beta_3$ integrins are all substantially compromised. For example, knockdown of CALDAG-GEF1 expression in human T cells affects LFA-1–mediated adhesion to ICAM-1, but not $\alpha_4\beta_1$ adhesion to vascular cell adhesion molecule-1, which is protein kinase C dependent[18]; in some circumstances the CALDAG-GEF1 and PKC pathways operate separately to activate mouse platelet $\alpha_{IIb}\beta_3$ (ref. 19).

*KINDLIN3* (the encoded protein is also known as FERMT3, URP2 or MIG-2B) has recently become a prime candidate as a causative gene for LAD-III because Kindlin-3–deficient mice show a Glanzmann's thrombasthenia–like phenotype[20] and have leukocytes with integrin function defects (see Moser *et al.*[21] in this issue of *Nature Medicine*). The *KINDLIN3* gene (chromosome 11: 63.73–63.75 Mb) is located 0.5 Mb from *CALDAGGEF1* at chromosome 11q13.1 in the region of shared haplotypes in the LAD-III–affected families in this study (**Supplementary Fig. 2**). Furthermore, Kindlin-3 is exclusively expressed by hematopoietically-derived cells[22]. It is homologous to the adhesion plaque protein Kindlin-1, which is expressed in epithelial cells, and also to Kindlin-2, which is widely expressed[22,23]. Loss-of-function mutations in *KINDLIN1* lead to Kindler syndrome, a human disease characterized by skin blistering due to integrin dysfunction[24,25]. The kindlins have a FERM (protein4.1-ezrin-radixin-moesin) domain highly homologous to the FERM domain of talin[26–28]. Kindlin-2 and

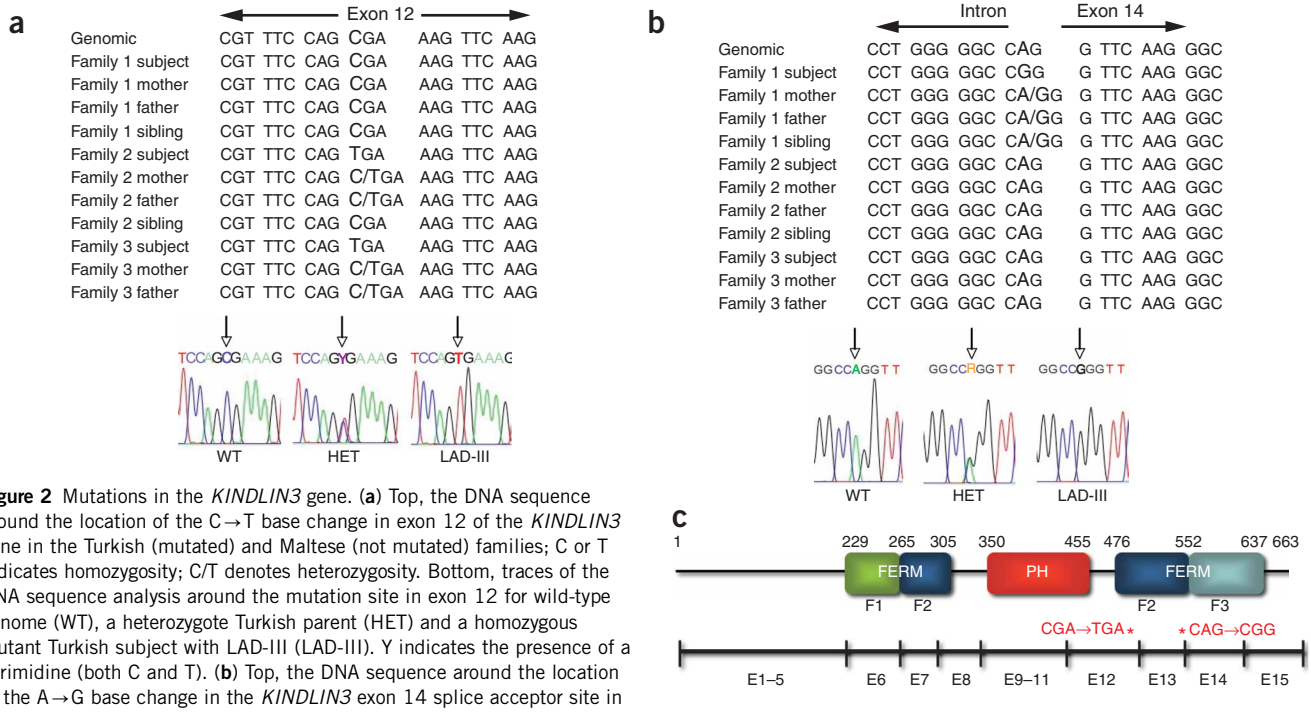


**Figure 2** Mutations in the *KINDLIN3* gene. (**a**) Top, the DNA sequence around the location of the C→T base change in exon 12 of the *KINDLIN3* gene in the Turkish (mutated) and Maltese (not mutated) families; C or T indicates homozygosity; C/T denotes heterozygosity. Bottom, traces of the DNA sequence analysis around the mutation site in exon 12 for wild-type genome (WT), a heterozygote Turkish parent (HET) and a homozygous mutant Turkish subject with LAD-III (LAD-III). Y indicates the presence of a pyrimidine (both C and T). (**b**) Top, the DNA sequence around the location of the A→G base change in the *KINDLIN3* exon 14 splice acceptor site in the Maltese (mutated) and Turkish (not mutated) families; A or G at this position indicates homozygosity; A/G denotes heterozygosity. Bottom, traces of the DNA sequence analysis around the mutation site in splice acceptor site of exon 14 for the WT, the heterozygote Maltese parents (HET) and the homozygous mutant Maltese subject with LAD-III; R indicates the presence of a purine (both A and G). More extensive original data for **a** and **b** are shown in **Supplementary Figure 5** online. (**c**) Diagrammatical representation of human KINDLIN-3 protein (residues 1–663), showing the three FERM subdomains (F1–3) characteristically intersected with a pleckstrin homology (PH) domain. The location of the exons encoding the different regions of the KINDLIN-3 protein is shown. The position of the two LAD-III mutations, one in exon 12 (E12) and the other affecting the join of E13 and E14 of the *KINDLIN3* gene, detailed above in **a** and **b**, respectively, are highlighted with red asterisks.

Kindlin-3 both bind to the integrin β subunit through their FERM domains; of note, the integrin activation-inducing activity of the talin FERM domain is enhanced by integrin-bound kindlins[20,29,30].

We screened the human *KINDLIN3* gene for germline mutations in the three subjects with LAD-III and their immediate families (**Fig. 2**). The two Turkish subjects with LAD-III (families two and three) were

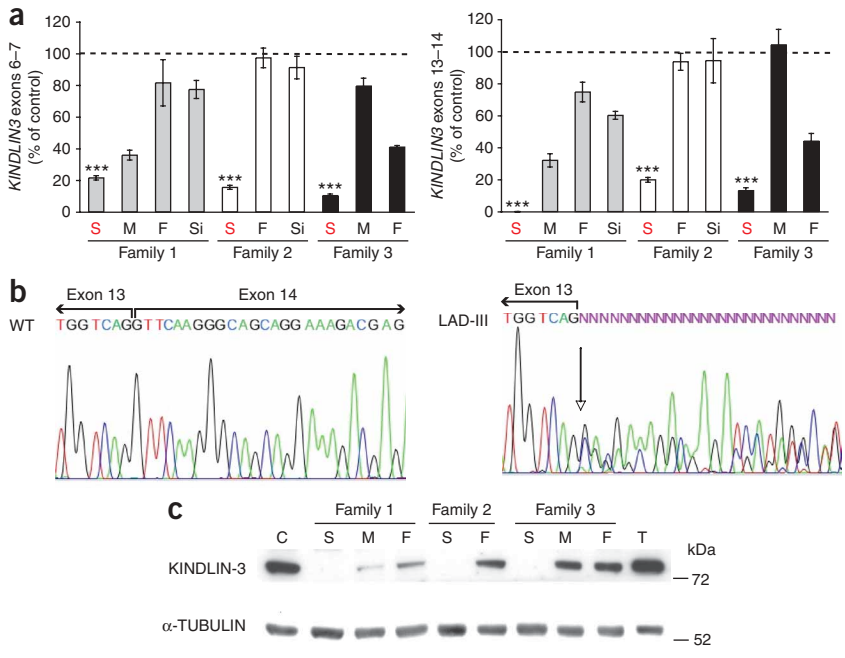


**Figure 3** Analysis of mutated *KINDLIN3* mRNA in subjects with LAD-III and their relatives. (**a**) Quantification of *KINDLIN3* mRNA by quantitative RT-PCR analysis with probes covering exons 6–7 and 13–14. Subjects and their relatives were compared to two control EBV-transformed cell lines (set at 100%). $n = 2$ for each family; $^{***}P < 0.001$. Si, sibling. (**b**) Effect of the splice acceptor site mutation in the Maltese subject on *KINDLIN3* mRNA at the exon 13-14 junction. The RT-PCR product encompassing the mRNA sequence specifying exons 12–15 was compared between control (WT) and the Maltese LAD-III subject. The unique DNA sequence specifying the end of exon 13 (last seven base pairs) and the start of exon 14 (25 base pairs) is shown for WT. For the Maltese subject with LAD-III, aberrant sequence is apparent after the last seven bases of exon 13 (arrow indicates beginning of aberrant sequence) which is consistent with an exon 14 splice acceptor mutation. (**c**) KINDLIN-3 protein abundance in each family, as detected in the B cells of the parents (M, F) but not of the LAD-III subjects (S). A nonrelated EBV transformed cell line (C) and primary T lymphoblasts (T) are included as controls. α-TUBULIN was used as a loading control.

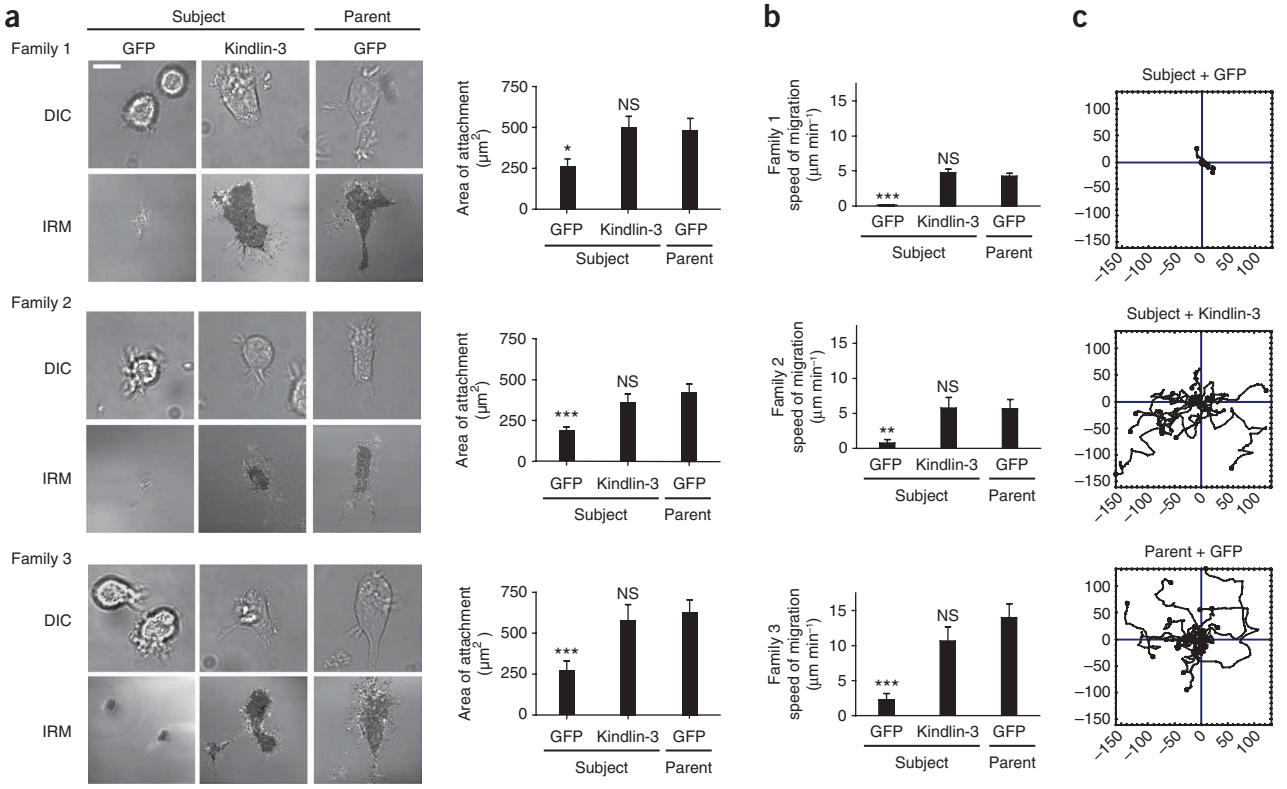


**Figure 4** Adhesion and migration characteristics of LAD-III B cells expressing wild-type Kindlin-3. (**a**) Left, DIC and IRM images of adhesion on ICAM-1 of LAD-III B cells transfected with either EGFP or a *Kindlin3* cDNA construct and compared with a parent's B cells (family 1 and 3, mother; family 2, father) transfected with EGFP ($n = 2$ for each cell type). Right, quantification of the area of close contact for $n = 35$ cells. Scale bar, 10 μm. Data are shown as means ± s.e.m.; *, $P < 0.05$, ***$P < 0.001$ and NS, not significant. (**b**) Migration characteristics of LAD-III B cells adhered to ICAM-1. B cells from subjects with LAD-III were transfected with either EGFP or wild-type EGFP-*Kindlin3* cDNA constructs and compared with EGFP-transfected parents' B cells (family 1 and 3, mother; family 2, father) (representative experiment of $n = 3$ for each family). Data are shown as means ± s.e.m.; **$P < 0.01$ and ***$P < 0.001$. (**c**) The cell-tracking results from an individual experiment with cells from the family two subject with LAD-III and from his father, transfected as in **b**.



homozygous for a C→T nonsense mutation at nucleotide 1525 in exon 12 that changes a CGA codon to a TGA termination codon (R509X; **Fig. 2a**). The parents were heterozygous for this change, and the healthy sister in family two was homozygous for the wild-type allele (**Fig. 2a**). This mutation lies within the amino acid sequence coding for the carboxy-terminal half of the FERM F2 subdomain and could have an impact on the integrin binding F3 subdomain (**Fig. 2c**).

In contrast to the Turkish families, all members of the Maltese family were homozygous for the wild-type C allele at nucleotide 1525. However, further screening of this family revealed an A→G mutation at the splice acceptor site of exon 14 in the *KINDLIN3* gene (**Fig. 2b**). The Maltese subject with LAD-III was homozygous for the G allele and the three other family members were heterozygous (**Fig. 2b**). This mutation, predicted to affect the correct splicing of exon 13 to exon 14, would also prevent the generation of an intact FERM F3 subdomain, thus having implications for the binding of KINDLIN-3 to integrin (**Fig. 2c**). The Turkish subjects' families were all homozygous for the wild-type A allele.

We used quantitative RT-PCR to investigate the effect of these two mutations on *KINDLIN3* mRNA expression in the transformed B cell lines. We used a probe specific for exons 6–7 (distant from the mutation sites) and one for exons 13–14 (designed to measure the integrity of the mRNA distal to the termination codon mutation in the Turkish subjects or in the neighborhood of the exon 14 splice acceptor site mutation in the Maltese subject). The parents of all three LAD-III–affected families had similar levels of *KINDLIN3* mRNA as detected with both sets of probes compared to normal controls (**Fig. 3a**). The two Turkish subjects with LAD-III expressed substantially lower levels of *KINDLIN3* mRNA, as detected by both probe sets (**Fig. 3a**), indicating that the C→T mutation that specifies a termination codon at nucleotide 1525 in exon 12 affects the stability of the *KINDLIN3* mRNA.

For the Maltese subject, *KINDLIN3* mRNA was undetectable by the exon 13–14 probes that covered the region where the A→G mutation was located (**Fig. 3a**). However, an RT-PCR assay covering exons 12–15 did generate a low level of product (**Fig. 3b**). Analysis of this product revealed a mixture of cDNAs, all of which contained a normal sequence up to the exon 13-14 boundary, followed by a variety of aberrant splice products (**Fig. 3b**). These results are consistent with the A→G mutation in the exon 14 splice acceptor site causing abnormal splicing of the exon 13 to exon 14 junction in the Maltese subject. However the cells of the Maltese subject also expressed lower levels of mRNA as detected using the exon 6 and 7 probes (**Fig. 3a**), suggesting that the A→G mutation, like the Turkish C→T mutation, destabilizes the *KINDLIN3* mRNA upstream of the mutation.

By western blot analysis, KINDLIN-3 was detectable in the parents' cells for all three families, but, as predicted from the low levels of

mRNA, was absent from the cells of subjects with LAD-III (**Fig. 3c**). Thus, two different mutations in the *KINDLIN3* gene had the effect of preventing expression of the KINDLIN-3 protein.

Kindlin-3 is crucial for activating integrins on mouse hematopoietic cells and for their adhesion-related functions[20,21]. However there has been no comparable study of human KINDLIN-3, and it was essential to test whether the defects in adhesion and migration shown by the cells from subjects with LAD-III could be repaired by the expression of the wild-type protein. Therefore, we transfected B cells from the three subjects with LAD-III with *EGFP-Kindlin3* cDNA that is able to reverse the integrin activation defect in Kindlin-3-deficient mouse cells[21,22]. As assessed by IRM, control EGFP-transfected LAD-III cells made poor contacts compared with EGFP-transfected parents' cells (**Fig. 4a**). In contrast, expression of the *EGFP-Kindlin3* cDNA in LAD-III B cells increased their adhesion to ICAM-1. Both IRM images and quantification of the areas of attachment provided evidence that Kindlin-3–transfected cells from the Maltese and Turkish subjects with LAD-III were able to make adhesions equivalent in area to the parents' cells (**Fig. 4a**).

To show further that expression of wild-type Kindlin-3 was able to overcome the LAD-III phenotype, we tested the same cells for their ability to undergo LFA-1–mediated migration on ICAM-1–coated surfaces. LAD-III cells from all three families expressing EGFP–Kindlin-3 migrated on ICAM-1 similarly to control EGFP-transfected parents' cells, whereas this was not the case for control EGFP-transfected LAD-III cells (**Fig. 4b** and **Supplementary Videos 1–4** online). B cells from the Maltese and Turkish subjects with LAD-III expressing wild-type Kindlin-3 also had an identical pattern of random motility compared to the parents' cells (**Fig. 4c** and data not shown).

In summary, we describe two independent disabling mutations in the *KINDLIN3* gene in subjects with LAD-III. The Maltese subject with LAD-III has a homozygous inactivating mutation within the splice acceptor site of exon 14, whereas both Turkish subjects contain an inactivating mutation in exon 12 resulting in a translational stop codon. This C→T nonsense mutation was recently described in three other Turkish subjects in a correspondence published after submission of this manuscript[31]. Both mutations lead to an overall decrease in *KINDLIN3* mRNA levels and loss of protein expression. Furthermore, the failure of leukocytes from subjects with LAD-III to adhere and migrate, as is typical of the LAD-III phenotype, is restored by expression of wild-type Kindlin-3.

Kindlin-3 binds the cytoplasmic tails of $\beta_1$, $\beta_2$ and $\beta_3$ integrins at the membrane distal NPXY motif[20,21,29]. The evidence suggests that the binding of Kindlin-3 enhances talin binding at the membrane-proximal NPXY site, which causes an increase in integrin affinity[32,33] Further details of the relationship between Kindlin-3 and integrin activation remain to be investigated. However, the implication of our results is that KINDLIN-3 is essential for the generation, maintenance or both of integrin activity on human leukocytes and platelets and that this crucial step is faulty in individuals with LAD-III.

A distinguishing feature between individuals with LAD-III and individuals with other integrin deficiency syndromes is the normal expression, but lack of function, of the $\beta_1$, $\beta_2$ and $\beta_3$ integrins expressed by their leukocytes and platelets. This failure of integrin function leads to immune deficiency and bleeding problems[8]. To date, 14 individuals with LAD-III have been reported, of which the majority are of Turkish origin[10–12,34,35]. The nature of the disabling mutation(s) has been a focus of interest for more than a decade since the first reports of these individuals. In this study we identify mutations in the *KINDLIN3* gene in the subjects with LAD-III and show that expression of wild-type Kindlin-3 can overcome the LAD-III defects by generating integrin adhesive contacts and integrin-mediated migration of LAD-III lymphocytes. This provides strong biological evidence for the presence of inactivating mutations in the *KINDLIN3* gene as a cause of the LAD-III phenotype.

## METHODS

**Subjects with LAD-III.** All affected children in the three families under study showed characteristics typical of the LAD-III disorder; cerebral hemorrhages at birth (presumed to result from a failure of platelet integrin function), an increased leukocyte blood count (presumed to result from the difficulty of leukocytes exiting the circulation as a result of a lack of leukocyte $\beta_1$ and $\beta_2$ integrin function) and recurrent tissue infections. In family one, the female Maltese subject was previously characterized as having LAD-III[11] (see **Supplementary Fig. 1**). This child was successfully bone marrow transplanted in November 2001 (ref. 11). In family two, the subject, a 4-year-old Turkish boy, was also previously identified as having LAD-III (family five in ref. 12). A female Turkish subject with LAD-III (family three) who shows clinical features in keeping with a LAD-III diagnosis has not been previously reported on. This subject (<1 year old) first presented with anemia, thrombocytopenia and leukocytosis (35–100 × $10^9$ cells per liter; neutrophilia and lymphocytosis in equal proportions). This subject had a history of delayed cord separation, hematuria, melena, petechias and severe infection. These symptoms all persisted, necessitating repeated erythrocyte transfusions. The subject has a clinical phenotype suggestive of the severe form of LAD-I: septicemia, axillary ulceration without pus collection, diffuse cellulitis on the right arm and delayed cord separation (surgical separation at day 20). Additionally, platelet aggregation tests showed abnormalities as seen in Glanzmann's thrombasthenia.

We obtained approval for studies with the newly reported human primary cells from the Local Ethical Committee of the SB Ankara Diskapi Children's Hospital, Ankara, Turkey. Additionally, informed consent was obtained from the donor families and control individuals.

**Antibodies and other reagents.** Monoclonal antibodies 38 (specific for $\alpha_L$ integrin, CD11a), TS1/18 (specific for $\beta_2$ integrin, CD18), HP2/1 (specific for $\alpha_4$ integrin, CD49d) and P5D2 (specific for $\beta_1$ integrin, CD29) have been previously reported[11,36]. We also used the following antibodies: rabbit polyclonal antibody to CALDAG-GEF1 (serum 3752) and DM 1A, an α-tubulin–specific monoclonal antibody (Sigma-Aldrich). We coupled a human-specific KINDLIN-3 peptide (EPEEELYDLSKVVLA; amino acids 156-170) to Imject maleimide activated keyhole limpet hemocyanin (Pierce) and used it to immunize rabbits. We prepared the affinity-purified antibodies as previously described[22].

We used cDNAs encoding human cytosolic Myc–CALDAG-GEF1 and membrane-localized CALDAG-GEF1 (RASGRP2-Flag)[15] and mouse cytosolic CALDAG-GEF1–Flag in this study. The mouse EGFP-Kindlin3 DNA construct has been described previously[22]. We prepared full-length ICAM-1-Fc protein as previously described[11].

**Cells and cell transfections.** We prepared T lymphocytes and expanded them in culture as previously described[11]. EBV-transformed B lymphoblastoid cells were derived from peripheral blood mononuclear cells of subjects and their relatives by Research Cell Services, Cancer Research UK, who derived these cells by standard procedures. We obtained standard control EBV-transformed cell lines from the same source. We maintained all cells in RPMI-1640 medium with 10% FCS.

We washed EBV-transformed B cells in OptiMEM + GlutaMAX (Invitrogen) and electroporated 2 × $10^7$ cells with 10 μg per reaction of *CALDAGGEF1* or *Kindlin3* cDNA constructs or 5 μg of EGFP-N1 (BD Biosciences) with a Gene Pulser with Capacitance Extender (Bio-Rad UK) set at 960 μF and 260 mV. We maintained the DNA-transfected cells in RPMI 1640 with 10% FCS for up to 24 h. We evaluated the efficiency of the transfection by flow cytometry, and we sorted the EGFP-positive cells with a MoFlo Cell Sorter (Beckman Coulter) before use in migration assays.

**Sequence analysis.** We analyzed genomic DNA from all subjects, relatives and nonrelated controls for sequence alterations in all exons and intron-exon boundaries of the *CALDAGGEF1* (reference genomic data from http://genome.ucsc.edu/ (chr11: 64,250,959–64,269,504)) and *KINDLIN3* (chr11: 63,730,782–63,747,930) genes by direct DNA sequencing in both orientations. Details and PCR conditions are available from the authors.

**Quantitative reverse transcription-PCR.** We quantified human *CALDAGGEF1* and *KINDLIN3* mRNA levels with TaqMan technology. Briefly, we extracted RNA from leukocytes with the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). We reverse-transcribed the RNA with the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies) and purified it with the QIAquick PCR Purification Kit (Qiagen). We amplified a total of 40 ng of cDNA per reaction by TaqMan Gene Expression Assays from Applied Biosystems: for *CALDAGGEF1,* Hs00183378_m1 (exons 8–9) and Hs01057123_m1 (exons 2–3); for *KINDLIN3*, Hs00258828_m1 (exons 13–14) and Hs01075695_m1 (exons 6–7); and for control genes: Hs99999905_m1 (*GAPDH*) and Hs00202782_m1 (*SF3B1*).

We analyzed the samples on the ABI 7900HT Sequence Detection System instrument. We ran each sample in quadruplicate and expressed the data as a function of threshold cycle ($C_T$). We corrected the $C_T$ values for reactions amplifying *CALDAGGEF1* or *KINDLIN3* by the $C_T$ value for two control housekeeping genes, *SF3B1* and *GAPDH* to give $\Delta C_T$. The difference in $\Delta C_T$ values between test and control cDNA samples allowed us to quantify the relative expression of the gene as follows: 2^– [($\Delta C_T$ test – $\Delta C_T$ control) – ($\Delta C_T$ control – $\Delta C_T$ control)]

**Video microscopy.** We coated either 35-mm glass-bottom microwell dishes (MatTek) or ibiTreat μ-Slides VI (Thistle Scientific) overnight with 3 μg ml$^{-1}$ ICAM-1-Fc and then blocked them with BSA. We allowed the lymphocytes ($4 \times 10^5$ cells per ml in HBSS with 20 mM HEPES buffer) to settle for 10 min at 37 °C and then removed the nonattached cells with gentle washing. We took images with a Nikon Diaphot 300 microscope, using a 20× or 63× lens and AQM$^{2001}$ Kinetic Acquisition Manager software (Kinetic Imaging). We tracked the cells at 15-s intervals with Motion Analysis software (Kinetic Imaging) and analyzed the data with a Mathematica notebook (Wolfram Research) developed by D. Zicha (Cancer Research UK).

**Interference reflection microscopy.** We plated EBV-transformed B cells on ibiTreat μ-Slides coated with 3 μg ml$^{-1}$ ICAM-1. We acquired images of close substrate contact of the migrating cells between 10 min and 30 min of attachment to ICAM-1 with a Zeiss Axiovert 100 M inverted confocal microscope with a 63 × NA1.4 Plan-Apochromat oil-immersion objective lens. For evaluation of adhesion status, we measured the area of contact with MetaMorph Offline 7.1 ($n = 35$ cells per sample type).

**Statistical analyses.** The adhesion and migration assays are presented as the means ± s.e.m. We used the unpaired Student's $t$ test on the data with GraphPad Prism software version 4 for Macintosh computers. We analyzed the quantitative RT-PCR data by two-way analysis of variance. We separately assessed the data for each family and compared each subject with his or her relatives. Significant differences are indicated as follows: $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

**Additional methods.** Detailed methodology is described in **Supplementary Methods** online.

*Note: Supplementary information is available on the Nature Medicine website.*

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AUTHOR CONTRIBUTIONS

L.S., K.H., I.T., M.F. and N.H. designed the experiments; L.S., K.H., A.M., I.P. and R.E. performed the experiments; and A.M. provided subject blood samples; S.U. provided the antibody to Kindlin-3; M.M. provided the EGFP–Kindlin-3 construct;. L.S., K.H., A.M., I.P., M.F., I.T. and N.H. were involved in data analyses. All authors contributed to the writing or editing of the manuscript. N.H. supervised the project and wrote the initial manuscript.

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