Defective binding of ETS1 and STAT4 due to a mutation in the promoter region of *THPO* as a novel mechanism of congenital amegakaryocytic thrombocytopenia

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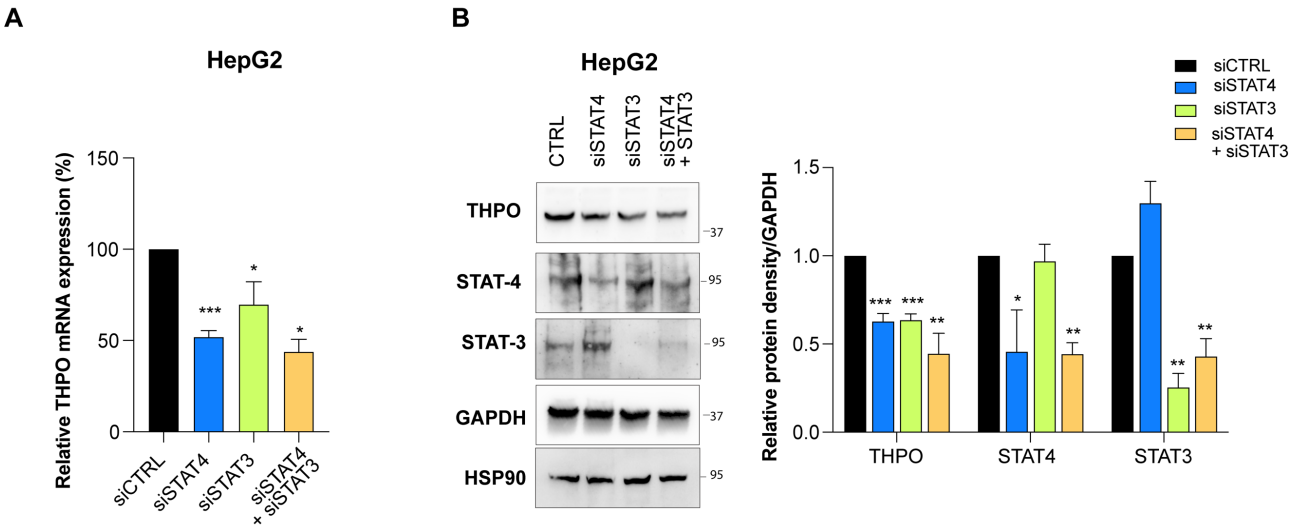
SUPPLEMENTARY INFORMATION

Supplementary FIGURE 1



Supplementary Figure Legends

Figure 1 STAT4 and STAT3 regulates THPO expression

A THPO expression was evaluated by RT-qPCR, normalized to beta-Actin RNA expression levels, in HepG2 cells upon silencing endogenous STAT4 and STAT3 with specific siRNA for 48h.

B Western blot analysis of THPO, STAT4 and STAT3 expression using HSP90 and GAPDH as loading control in cell lysates of HepG2 cells. Right: graph shows the quantification of Western blot bands measured by densitometry, normalized to GAPDH.

Supplementary Methods

Cell culture and Transfection

HEK293T and HepG2 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and with penicillin and streptomycin (100 IU/mL each). Cell lines were maintained in a 37°C, 5% CO_2 incubator.

Cells were transfected when the culture reached 50-80% confluence level. For DNA transfections, the appropriate amount of DNA, was used together with Lipofectamine 2000 (Invitrogen™, Life Technologies, Carlsbad, CA, USA, #11668019) following manufacturer's instructions; for siRNA transfections, cells were transfected with 100 nM siRNA oligonucleotides with Lipofectamine RNAiMax (Invitrogen™, #13778075) following manufacturer's instructions; as a negative control, the Eurofins Control siRNA was used. Target siRNA sequences were siSTAT4-5'-AAGGCAATTGGAGAAACTA-3' (as previously reported by[1]), siETS1 5'-ACUUGCUACCAUCCCGUAC-3' (as previously reported by [2]).

RNA extraction and Quantitative Real-Time PCR

total RNA was extracted with High Pure RNA Isolation Kit (Roche, #11828665001). RNA concentration, quality and purity were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). Aliquots of 1 µg of total RNA were retrotranscribed with iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, #1725037), and the analyzed genes were amplified using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, 1725120) on Applied Biosystems 7500 FAST DX. Data were analyzed with the 7500 Fast Real-Time PCR System Software v2.3. Experiments were performed at least three times, and each sample is the average of a technical duplicate. The quantification is based on the $2^{-\Delta\Delta Ct}$ method using the proper housekeeping gene levels as normalization reference. Primers sequences are reported in Supplementary Table 2.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as previously described [3]. Chromatin was immunoprecipitated with the STAT4 or ETS-1 antibody (Supplementary Table 3). IgGs purified from rabbit serum were used as negative control. Co-immunoprecipitated DNA was analyzed by real-time PCR. Promoter occupancy was calculated as percent of input chromatin immunoprecipitated using the $2^{-\Delta Ct}$ method. Primers sequences are reported in Supplementary Table 2.

Western Blot and Co-immunoprecipitation analysis

Total cell extracts were prepared in lysis buffer (300 mM NaCl, 50 mM Tris-HCl pH7,5, 1 mM EDTA, 1% NP-40) supplemented with 1 mM PMSF, 5 mM NaF and 1 mM Na_3VO_4. Protein concentration was determined with Bio-Rad Protein Assay Reagent (Bio-Rad, #500-0006). Lysates were resolved

by SDS/PAGE and transferred to nitrocellulose membranes (Bio-Rad, #1704158). Western blot was performed according to standard procedures.

Co-IP experiments with endogenous proteins were performed using Co-IP buffer (150 mM NaCl, 50 mM Tris-HCl pH8, 1 mM EDTA, 1% NP40) supplemented with protease inhibitors as described before. Samples were cleared by centrifugation for 5 min at 13,000g at 4°C and incubated for 3h at 4°C with anti-STAT4 antibody. After 1 h of incubation with protein A/G PLUS Agarose (Santa Cruz Biotechnologies sc-2003), immunoprecipitates were washed three times in Co-IP buffer, resuspended in Laemmli sample buffer, and analysed by western blotting.

The antibodies used are listed in Supplementary Table 3. Anti-mouse and anti-rabbit HRPO-conjugated (Santa Cruz) antibodies were used as secondary antibodies. Images were acquired using ChemiDoc MP Imaging System (Bio-Rad), intensity of the bands was quantified using FIJI software (NIH Image) [4].

Dual Luciferase Assays

HEK293T cells were seeded in 24-well plates and transfected with 100 nM of Control, STAT4, ETS1 siRNAs alone or in combination. After 24 hours, cells were transfected with 300 ng of pGL3-THPO either wild-type or c.-323C>T reporter vectors, and 100 ng of pRL-CMV. 6 hours after transfection, medium was changed and 18 hours later luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega, #E1910) on a Glomax Discover (Promega). Relative Luciferase Units (RLU) were calculated by normalizing the luciferase units measured for the Firefly luciferase on the luciferase units of the Renilla luciferase in each sample.

Proximity Ligation Assay

HepG2 cells were seeded on coverslips, after 48h the cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature, followed by two washes with PBS. The cells were permeabilized with 0.1% Triton X-100. The PLA was performed using the Duolink In Situ Red Starter Kit Mouse/Rabbit (Sigma) according to the manufacturer's protocol. The anti-STAT4 and anti-ETS1 primary antibodies were used diluted 1:50. The stained coverslips were mounted on slides and visualized Zeiss Axioplan 2 epifluorescence imaging microscop. Representative images are shown for each biological group.

Methods References

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2. Zhu Q, Ren H, Li X, et al. Silencing KIF14 reverses acquired resistance to sorafenib in hepatocellular carcinoma. Aging (Albany NY) 2020;12(22):22975–23003.
3. Capaci V, Bascetta L, Fantuz M, et al. Mutant p53 induces Golgi tubulo-vesiculation driving a prometastatic secretome. Nat Commun 2020;11(1):3945.

4. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012;9(7):676–82.

Supplementary Table1. List of candidate genes analyzed in mutational screening:

ABC1, ABCA1, ABCD4, ABL, ABL1, ACAD9, ACD, ACP5, ACTN1, AD2, ADA, ADAMTS13, ADAR, AGK, AGMX1, AGS1, AGS2, ALG8, AML1, ANKRD11, ANKRD26, AP3B1, API3, APOE, APT1, APT1LG1, ARHGAP31, ARHGEF1, ARPC1B, ARVCF, AS, ASLN, ATP7B, ATRX, ATS, BCOR, BCR, BCR1, BF, BFD, BIRC4, BLOC1S6, BNSP, BRAF, BRCA1, BRCA2, BRIP1, BTBD12, BTK, BTNL2, C10orf24, C13orf12, C16orf57, C17orf68, C1QA, C1R, C20orf41, C3, C3orf64, C6orf209, C6orf25, C7orf5, C7orf6, C9orf102, C9orf8, CA2, CALR, CASP10, CBFA2, CD109, CD19, CD20, CD36, CD36L2, CD40LG, CD46, CD49B, CD81, CDC42, CDC4L, CELIAC3, CFB, CFH, CFHL1, CFHL1P, CFHL3, CFHR1, CFHR1P, CFHR3, CFI, CHS1, CIITA, CLCN7, COG1, COG4, COG6, COL4A5, COMT, CORIN, CR2, CTC1, CTLA4, CYCS, D22S11, DCLRE1C, DFNA1, DFNA14, DFNA17, DFNA38, DFNA6, DGKE, DGUOK, DHFR, DIAPH1, DKC, DKC1, DLEU8, DLL4, DNAJC21, DNASE1, DNL1, DOCK6, EFL1, EFTUD1, ELA2, ELANE, EOGT, ERBB3, ERCC4, ERCC6L2, ESCO2, ETV6, EVI1, F8VWF, FACA, FACC, FACD, FACE, FANCA, FANCB, FANCC, FANCD, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCH, FANCI, FANCL, FANCM, FARS1, FARS2, FAS, FAS1, FASLG, FCG2, FCGR2, FCGR2A, FCGR2A1, FCGR2B, FCGR2C, FIP1L1, FLI1, FLN, FLN1, FLNA, FLT, FLT1, FOXP3, FRAP, FRAP1, FRAP2, FYB, FYB1, G1P1, G6PC3, GALC, GATA1, GATA2, GBA, GF1, GFI1B, GLBA, GLUC, GLVR2, GNA14, GP1B, GP1BA, GP1BB, GP2B, GP3A, GP9, GUC1A3, GUCY1A1, GUCY1A3, HDLDT1, HELLPAR, HF, HF1, HF2, HFL1, HFL2, HIGM1, HIRA, HLA-B, HLA-DR1B, HLA-DRB1, HLCS, HOX1, HOX1I, HOXA11, HPS5, HYOU1, ICOS, IDDM12, IF, IFI4, IFI41, IFI75, IFIH1, IFNG, IGKJRB1, IKZF1, IL7R, IMD1, IMD2, IMD3, IMD5, IPEX, IRAK1, IRF2BP2, ITGA2, ITGA2B, ITGB3, ITK, IVD, JAK2, JMJD1C, JMS, KDM6A, KIAA1161, KIAA1546, KIAA1596, KIAA1794, KIF15, KMT2D, KNSL7, KRAS, KRAS2, LARS2, LAT, LBR, LCCS2, LDLB, LIG4, LMBRD1, LMP2, LMP7, LOH18CR1, LOH19CR1, LPI, LRBA, LYP, LYST, MAD2L2, MAP2K1, MCP, MDS1, MECOM, MHC2TA, MIC10, MLL2, MLVAR, MMAA, MMAB, MMACHC, MMUT, MPIG6B, MPL, MRT5, MRX52, MS4A1, MTOR, MULK, MUT, MVK, MYH9, MYORG, MYSM1, NABP1, NBEAL2, NBN, NBS, NBS1, NFKB1, NFKB2, NHEJ1, NHP2, NIPBL, NOLA2, NOLA3, NOP10, NOS3, NOTCH1, NP, NPM1, NRAS, NS1, NSUN2, NUMA1, OBFC2A, OCLN, OCRL, OPD1, OPD2, OPN, OSTM1, PA, PAGA, PALB2, PARN, PCCA, PCCB, PDB2, PDGFB, PDGFR, PDGFRB, PEPD, PHF9, PHGDH, PLAU, PLDN, PML, PNP, POMP, PRDX1, PRF1, PRKACG, PRKAR1, PRKAR1A, PRKCD, PRKMK1, PSAP, PSMB4, PSMB8, PSMB9, PTPN11, PTPN22, PTPN8, PXMP1L, RAD51, RAD51A, RAD51C, RAD54, RAG1, RAG2, RARA, RASGRP1, RBM8, RBM8A, RBPJ, RBPSUH, RBS, RECA, RFWD3, RFX5, RFXANK, RFXAP, RNASEH2A, RNASEH2B, RNASEH2C, RPS19, RREB1, RTEL1, RUNX1, SALL4, SAMD9, SAMD9L, SAMHD1, SAP1, SAP2, SARS2, SARSM, SBDS, SC5D, SC5DL, SCARB2, SCIDA, SEC24C, SF3B1, SH2D1A, SIS, SLC19A2, SLC20A2, SLC35A1, SLC46A1, SLC7A7, SLFN14, SLX4, SMARCAL1, SMARCD2, SMPD1, SNX10, SP110, SPATA5, SPP1, SRC, SRC1, SRK, SRP54, STAT1, STAT3, STAT4, STAT5B, STIM1, STOX1, STT3B, STX11, TALDO1, TAN1, TAPA1, TBL1XR1, TBX1, TBXAS1, TCN2, TERC, TERT, TET2, TFRC, THBD, THC, THC2, THPO, TINF2, TMEM165, TNFAIP3, TNFRSF11A, TNFRSF13B, TNFRSF13C, TNFRSF6, TNFSF11, TNFSF12, TNFSF5, TNFSF6, TNRC21, TPP2, TREX1, TRIP8, TRMA, TSE1, TUBB1, TUPLE1, UBE2T, UFD1, UFD1L, UROS, USB1, USP18, UTX, VCF, VPS33A, VPS45, VPS45A, VPS45B, VWF, WAS, WASPIP, WDR79, WFS1, WIPF1, WND, WRAP53, XIAP, XPF, XPR1, XRCC2, XRCC9, ZAP70, ZBTB16, ZNF145, ZNFN1A1

Supplementary Table2. List of the oligonucleotides used in the present study.

Target:	**Primer sequence:**	**Direction:**
qPCR primers		
THPO transcript variant1	CTTCACAGCAGACTGAGCCA	FW
THPO transcript variant1	CTCCAGCAGAAGGGTCACTG	REV
STAT3	CATCCTGAAGCTGACCCAGG	FW
STAT3	AGGTCGTTGGTGTCACACAG	REV
STAT4	ACTTGAGCACTGCCTGGGAC	FW
STAT4	TTAGAAGCTGCCTCCCAGTCTT	REV
ETS1	GTCCCTTACTCAGCGCCTC	FW
ETS1	CCAAAAGGGGTAGCAAGGTCT	REV
Actin	CGCCGCCAGCTCACCATG	FW
Actin	CACGATGGAGGGGAAGACGG	REV
H3	GAAGAAACCTCATCGTTACAGGCCTGGT	FW
H3	CTGCAAAGCACCAATAGCTGCACTCTGGAA	REV
pri-miR-30d	GTGAGGGGAACAGGAAGTGG	REV
Cloning Primers:		
THPO wt	CATGGTACCATGTGGGCAATATCCGT	FW
THPO wt	CATAAGCTTCTGCCCAATCAGAGAAG	REV
THPO -323C>T	GCGTCACTTCTGGGGGCCTTCA	RV
THPO -323C>T	TGAAGGCCCCCAGAAGTGACGC	FW
ChIP Primers:		
pGL3-THPO	GTTGCCCATGTCCAGGAAAAG	FW
pGL3-THPO	AGTGTCTAAGCTTTCTGCCCAA	REV
pGL3-OFF	GTGAGCAAAAGGCCAGCAAA	FW
pGL3-OFF	ATAGTCCTGTCGGGTTTCGC	REV

Supplementary Table3. List of the antibodies used in the present study.

Target protein name:	**Producer:**	**ID number:**	**WB Dilution:**
ETS-1	GeneTex	GTX100639, RRID:AB_10632183	1:1000
GAPDH	Santa Cruz Biotechnology	sc-47724, RRID:AB_627678	1:5000
HSP90 alpha/beta (F-8)	Santa Cruz Biotechnology	sc-13119; RRID: AB_675659	1:5000
STAT3 (F-2)	Santa Cruz Biotechnology	sc-8019; RRID:AB_628293	1:1000
STAT4 (C-4)	Santa Cruz	sc-398228, RRID:AB_2810272	1:500
Thrombopoietin	Invitrogen	PA5-80125; RRID: AB_2747239	1:1000
Mouse normal IgG	Santa Cruz Biotechnology	sc-2025; RRID: AB_737182	-
Rabbit normal IgG	Santa Cruz Biotechnology	sc-2027; RRID: AB_737197	-
II anti mouse	Santa Cruz Biotechnology	sc-51602, RRID:AB_626603	1:2000
II anti rabbit	Santa Cruz Biotechnology	sc-2004, RRID:AB_631746	1:2000