Supplementary information

Nucleolin as activator of TCF7L2 in human hematopoietic stem/progenitor cells

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Supplementary Materials and Methods

Cells and culture conditions

MPB HSPCs from patients with non-hematological malignancies collected, CD34-immunopurified and frozen for subsequent use in autologous HSC transplantation, were described.\textsuperscript{3} Aliquots of cryopreserved HSPCs from deceased patients designated to be discarded were utilized after informed consent of legal guardians and in accordance with the Ethics Committee of the Medical Faculty of the Heinrich Heine University of Düsseldorf, as described.\textsuperscript{3,4} CD34+ cell purity was >90%. In Figure S1, aliquots of lysates from previously described cells were used, transduced with lentivirus encoding for full-length human nucleolin (HSPC-NCL), or with lentivirus encoding for N-terminally truncated nucleolin aa 289-709 (HSPC-NCL-289-709), or with lentivirus devoid of nucleolin cDNA (HSPC-mock).\textsuperscript{4} The lysates were from cell populations containing ~90% pure transduced viable cells.

In experiments where quantities of HSPCs were not sufficient, CD34+CD133+ cell line Mutz2, derived from PB of an acute myelogenous leukemia (AML) patient,\textsuperscript{51} was used as described.\textsuperscript{3} Mutz2 cells (ACC-271, DSMZ, Braunschweig, Germany) were cultured in \(\alpha\)-MEM...
supplemented with 20% FCS and 20% conditioned medium from 5637 cells (ACC-35, DSMZ). Cells were recently authenticated and tested for mycoplasma contamination.

Electrophoretic mobility shift assay (EMSA) and ChIP assay

TCF7L2 gene promoter upstream of the translation start site was described in the previous study. A search of this promoter (GenBank accession number AF522996) for potential nucleolin binding sites revealed the presence of two fragments that included the following sequences: nucleotides (nt) -1215 to -1208 (5'-TGAAATGA-3') and nt -427 to -420 (5'-TGAACCTGA-3'). Double-stranded oligonucleotides from the TCF7L2 promoter used in EMSA contained nt -1232 to -1193 of the TCF7L2 promoter (TCF7L2-A) or nt -440 to -406 (TCF7L2-B). The respective derivatives of the oligonucleotides TCF7L2-A and TCF7L2-B were devoid of nt -1213 to -1209 (Mut TCF7L2-A), or nt -425 to -421 (Mut TCF7L2-B). Double-stranded oligonucleotide carrying two copies of the sequence 5'-CCCTTGATCTTACC-3' that contains the optimal TCF binding motif was used as control oligonucleotide. Nucleolin-glutathione S-transferase (GST) fusion protein, comprising amino-acid residues 289-709 of nucleolin, and use thereof in EMSA was described previously.

ChIP experiments with protein A/G MicroBeads (Miltenyi Biotec, Gladbach, Germany) followed a protocol provided by Miltenyi Biotec, as described. Antibody specific for N-terminal peptide of nucleolin, affinity-purified on the peptide column, was used and controls included presaturation of this antibody with the blocking peptide. Immunoprecipitated DNA was quantified by real-time PCR. Primer sequences are summarized in Table S2.

Western blotting

Immunoblot analysis followed standard procedures, and normalized band intensity is shown as a percentage of cells nucleofected with empty expression vector (Figures 2B and S2) or of the HSPC-mock sample (Figure S1). Antibodies, validated previously, were as follows: antibody specific for N-terminal peptide of nucleolin, purified on the peptide column, and commercially available antibodies described in Table S3.
Luciferase reporter assays

The following TCF7L2 promoter reporter constructs, cloned into pGL4.10 reporter vector (Promega, Madison, WI, USA), were used: TCF7L2-WT-pGL4.10 construct, containing the full-length wild-type human TCF7L2 promoter\(^{54}\) (see also Supplementary Methods, page 2), and a derivative lacking nt -1213 to -1209 and -425 to -421 (TCF7L2-Mut-pGL4.10). CMV promoter-driven expression constructs contained the following: i) cDNA encoding for N-terminally FLAG-tagged full-length human nucleolin or ii) cDNA encoding for FLAG-tagged nucleolin amino-acid residues 289-709. Mutz2 cells were nucleofected with Nucleofector Solution-V (Lonza, Cologne, Germany) and luciferase measurements were performed as described.\(^{53}\) In Figure 2B, the cells were co-nucleofected with increasing amounts of full-length nucleolin expression vector (none, 0.2, 0.4, 0.6, 0.8 and 1.0 µg) together with the TCF7L2-WT-pGL4.10 reporter construct, and cell aliquots were used for analyzes shown.

Gene set enrichment analysis (GSEA)

GSEA was performed using GSEA v2.2 software (Broad Institute, Cambridge, MA, USA),\(^{56}\) and statistical difference was determined by 1000 gene set permutations, timestamp seed for permutation. Minimum gene set size was set to 15; max_probe was used to collapse multiple probe sets / gene. The nucleolin-dependent gene expression profile, comprising data obtained with MPB HSPCs from 3 patients (HSPC-NCL cells versus HSPC-mock control cells), was described and the necessary controls, including the validation of differential gene expression by qRT-PCR, were reported.\(^4\) Nucleolin protein levels were ~4-fold higher in HSPC-NCL versus HSPC-mock cells.\(^4\) Other gene sets used for GSEAs were as follows: in Figure 1A, gene ontology gene set regulation of Wnt signaling pathway and, in Figure 1B, TCF7L2-bound gene set, derived from human CD34+ MPB HPCs.\(^7\)

Supplementary Discussion

Wnt signals are relevant to regulation of HSPCs and the strength of Wnt signaling regulates hematopoiesis.\(^8,9,57\) On the other hand, deregulated Wnt signaling, linked to worse clinical
outcome for a subset of human cancers, is causatively associated with leukemogenesis and is of importance to the properties of LSCs. The effect of nucleolin on Wnt signaling regulators including TCF7L2 suggests its relevance to regulation of Wnt signaling.

TCF7L2 (also known as TCF4) is upregulated in HSCs/HSPCs and is capable of enhancing GATA2-mediated transcriptional activation. Furthermore, TCF7L2 is involved in hematopoietic regeneration and differentiation and cooperates with lineage master regulators to affect expression of key hematopoietic genes. On the other hand, its expression is elevated in CD34+ hematopoietic cells from patients in chronic myeloid leukemia (CML) blast crisis and TCF7L2 has a role in transcriptional changes in CML. In addition, overexpression of TCF7L2 in mantle cell lymphoma-initiating cells is associated with aberrant Wnt activity critical for their maintenance and survival. Additionally, installation of a cancer-promoting Wnt/SIX1 signaling axis by the MLL-AF9 oncoprotein in AML LSCs involves TCF7L2. TCF7L2 participates as well in aberrant Wnt activity in certain non-hematological malignancies.

TCF7L2 is a member of the TCF/LEF family of DNA-binding nuclear factors that are effectors of the Wnt signaling pathway, and interaction of β-catenin with TCF7L2 leads to activation of TCF7L2-bound genes. In addition, this interaction also serves as a target for cancer therapy. The herein-reported finding that nucleolin, as a TCF7L2 promoter-binding factor, upregulates TCF7L2 is novel and, in line with our published data, nucleolin as well elevates the level of N-terminally dephosphorylated β-catenin that, as was demonstrated by Staal and colleagues, transduces Wnt signals. Thereby, the signature of TCF7L2-bound genes is enriched by nucleolin (Figure 1B). Furthermore, genes bound by TCF7L2 are overrepresented among the upregulated genes encoding regulators of Wnt signaling. This includes BAMBI, LRP4, MDFIC and SOX4, that were shown to be TCF7L2-occupied and modulatable via TCF7L2 and β-catenin in non-HSPCs. Thus, transcriptional upregulation of Wnt signaling regulators by nucleolin partially involves TCF7L2.
Acknowledgments

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Supplement Table Legends

**Supplementary Table S1.** (A) Genes from the gene ontology gene set regulation of Wnt signaling pathway, upregulated by nucleolin in CD34+ MPB HSPCs. (B) Genes from the gene ontology gene set regulation of Wnt signaling pathway, downregulated by nucleolin in CD34+ MPB HSPCs. Nucleolin-dependent expression profile that comprised data obtained with HSPCs from 3 patients was described and the necessary controls, including the validation of differential gene expression by qRT-PCR, were reported.4 Criteria for listing differentially expressed genes in the Table are as follows: adjusted p-value ≤0.01 and fold change cutoff ≥2. Genes are ranked by adjusted p-value. Genes that are bound by TCF7L2, within 4 kilobases upstream or downstream of gene body, in human CD34+ MPB HPCs7 are indicated.

**Supplementary Table S2.** Sequences of primers used.

**Supplementary Table S3.** Antibodies used for immunoblotting.

Supplement Figure Legends

**Supplementary Figure S1.** Immunoblot analysis of lysates from HSPC-NCL, HSPC-NCL-289-709 and HSPC-mock cells. Top, analysis of the levels of TCF7L2. Bottom, analysis of the levels of N-terminally dephosphorylated β-catenin,S21 n=3. Nucleolin protein levels were
monitored by immunoblotting and were ~4-fold higher in HSPC-NCL, versus HSPC-NCL-289-709 and HSPC-mock cells (not shown), see also reference 4.

**Supplementary Figure S2.** After the measurement of the activity of TCF7L2 promoter, aliquots of samples from Figure 2C, left were used for immunoblot analysis of the levels of nucleolin and FLAG-tagged nucleolin.
| Gene symbol | Entrez gene ID | P-value          | Adjusted p-value | Fold change (upregulated) | Bound by TCF7L2 |
|-------------|---------------|------------------|------------------|--------------------------|----------------|
| TBL1XR1     | 79718         | 2.2e-07          | 4.4e-05          | 2.6                      | +              |
| MACF1       | 23499         | 2.4e-07          | 4.5e-05          | 2.3                      | +              |
| DAB2IP      | 153090        | 2.7e-07          | 4.8e-05          | 2.6                      | +              |
| FGF9        | 2254          | 4.0e-07          | 5.6e-05          | 17.1                     | +              |
| APP         | 351           | 6.9e-07          | 7.1e-05          | 4.0                      | +              |
| PTPRU       | 10076         | 7.1e-07          | 7.1e-05          | 8.5                      | -              |
| DISC1       | 27185         | 1.5e-06          | 9.9e-05          | 2.4                      | +              |
| NOG         | 9241          | 2.6e-06          | 1.3e-04          | 90.0                     | -              |
| DIXDC1      | 85458         | 3.6e-06          | 1.4e-04          | 5.1                      | +              |
| UBR5        | 51366         | 5.1e-06          | 1.7e-04          | 2.8                      | +              |
| MAPK14      | 1454          | 8.9e-06          | 2.3e-04          | 2.2                      | +              |
| CSNK1E      | 1454          | 1.6e-05          | 3.0e-04          | 2.7                      | -              |
| RECK        | 8434          | 1.7e-05          | 3.1e-04          | 4.8                      | -              |
| SOX4        | 6659          | 1.8e-05          | 3.2e-04          | 3.1                      | +              |
| STK3        | 6788          | 2.3e-05          | 3.7e-04          | 3.3                      | +              |
| TNKS2       | 80351         | 2.2e-05          | 3.7e-04          | 2.5                      | +              |
| RNF220      | 55182         | 2.5e-05          | 3.9e-04          | 2.2                      | -              |
| LRRK1       | 79705         | 2.8e-05          | 4.2e-04          | 2.3                      | -              |
| TNKS        | 8658          | 2.9e-05          | 4.3e-04          | 2.4                      | +              |
| LGR4        | 55366         | 4.0e-05          | 5.1e-04          | 4.3                      | +              |
| FZD7        | 8324          | 4.6e-05          | 5.5e-04          | 4.9                      | -              |
| LRP4        | 4038          | 5.5e-05          | 6.2e-04          | 2.7                      | +              |
| TERT        | 7015          | 5.9e-05          | 6.4e-04          | 7.6                      | -              |
| NPHP3       | 27031         | 7.2e-05          | 7.2e-04          | 2.0                      | -              |
| CCNY        | 219771        | 8.4e-05          | 7.9e-04          | 3.2                      | +              |
| FOXO1       | 2308          | 8.7e-05          | 8.0e-04          | 2.3                      | +              |
| ZBED3       | 84327         | 1.4e-04          | 1.1e-03          | 7.1                      | -              |
| CDK14       | 5218          | 1.4e-04          | 1.1e-03          | 3.1                      | -              |
| BAMBI       | 25805         | 1.6e-04          | 1.2e-03          | 8.9                      | +              |
| TLR2        | 7097          | 2.1e-04          | 1.4e-03          | 8.7                      | -              |
| VPS35       | 55737         | 2.4e-04          | 1.5e-03          | 2.2                      | -              |
| SMURF2      | 64750         | 2.7e-04          | 1.6e-03          | 3.2                      | -              |
| IBP2        | 3485          | 2.8e-04          | 1.6e-03          | 5.7                      | -              |
| AMER1       | 139285        | 4.6e-04          | 2.3e-03          | 5.3                      | -              |
| Gene     | Start | End   | p-value   | q-value | Direction |
|----------|-------|-------|-----------|---------|-----------|
| TCF7L2   | 6934  | 6934  | 4.7e-04   | 2.4e-03 | +         |
| CITED1   | 4435  | 4435  | 5.8e-04   | 2.7e-03 | -         |
| INVS     | 27130 | 27130 | 7.5e-04   | 3.3e-03 | -         |
| MDFIC    | 29969 | 29969 | 7.9e-04   | 3.4e-03 | +         |
| IFT20    | 90410 | 90410 | 8.1e-04   | 3.4e-03 | -         |
| RBPJ     | 3516  | 3516  | 1.2e-03   | 4.5e-03 | +         |
| TMEM88   | 92162 | 92162 | 1.5e-03   | 6.3e-03 | +         |
| MDFI     | 4188  | 4188  | 2.8e-03   | 8.6e-03 | -         |
## Supplementary Table S1B

| Gene symbol | Entrez gene ID | P-value | Adjusted p-value | Fold change (downregulated) | Bound by TCF7L2 |
|-------------|----------------|---------|------------------|-----------------------------|----------------|
| APOE        | 348            | 2.7e-07 | 4.8e-05          | 10.8                        | -              |
| LRP1        | 4035           | 1.1e-06 | 8.7e-05          | 4.8                         | -              |
| EGR1        | 1958           | 1.3e-06 | 9.3e-05          | 13.2                        | -              |
| CTNND1      | 51588          | 1.7e-06 | 1.0e-04          | 4.3                         | +              |
| FUZ         | 80199          | 9.2e-06 | 2.3e-04          | 3.1                         | -              |
| SRC         | 6714           | 1.6e-05 | 3.1e-04          | 5.7                         | -              |
| TLE1        | 7088           | 4.8e-05 | 5.7e-04          | 2.9                         | +              |
| KANK1       | 23189          | 1.4e-04 | 1.0e-03          | 2.0                         | +              |
| NFATC4      | 4776           | 1.8e-04 | 1.3e-03          | 4.6                         | -              |
| CAPRIN2     | 65981          | 2.4e-04 | 1.5e-03          | 2.0                         | -              |
| TIAM1       | 7074           | 3.3e-04 | 1.8e-03          | 2.2                         | +              |
| IGFBP4      | 3487           | 5.6e-04 | 2.7e-03          | 3.1                         | -              |
| SOX17       | 64321          | 1.8e-03 | 6.0e-03          | 2.1                         | +              |
| WNT5B       | 81029          | 1.8e-03 | 6.2e-03          | 6.4                         | -              |
| Amplified gene region | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') |
|-----------------------|---------------------------------|-------------------------------|
| TCF7L2 promoter       | CGATCCCCCTTTTCTATCTGTAATC       | AGCCGAAGATAACAGGAGGTG          |
| TCF7L2 intron 3       | AATTTTGGCCCTTTGGGATG            | ATTCTTTGGTTTGTGTCAGCC          |
**Supplementary Table S3**

| Antibody                                      | Vendor                                | Catalogue number |
|------------------------------------------------|---------------------------------------|------------------|
| anti-TCF7L2                                    | Santa Cruz, Dallas, TX, USA           | sc-271288        |
| anti-N-terminally dephosphorylated, active β-actin | Merck Millipore, Billerica, MA, USA   | 05-665           |
| anti-FLAG                                     | Sigma-Aldrich, St. Louis, MO, USA     | A8592            |
| anti-β-actin                                   | Sigma-Aldrich, St. Louis, MO, USA     | A1978            |
Supplementary Figures

Supplementary Figure S1

TCF7L2

Dephospho-β-catenin

β-actin
Supplementary Figure S2

| Control vector | + | - | - |
| Full-length nucleolin expression vector | - | + | - |
| Nucleolin 289-709 expression vector | - | - | + |

95 kDa

Nucleolin

95 kDa

FLAG-tagged nucleolin (FLAG-tag Ab)

55 kDa

β-actin