Novel regulators of megakaryopoiesis: The road less traveled by
Zeddies, S.

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ATAXIN2 controls megakaryocyte protein homeostasis and proper platelet functionality


*Department Hematopoiesis, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
^Department Blood Cell Research, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands
# Experimental Neurology, Goethe University Medical School, Frankfurt am Main, Germany
Abstract:

Multinucleated megakaryocytes accumulate large quantities of mRNA encoding, among others, proteins associated with the cytoskeleton, various platelet-specific granules, and membranes. To produce thousands of functional platelets per megakaryocyte, protein synthesis in the maturing megakaryocyte is likely to require strict spatio-temporal control. We observed that the RNA-binding protein ATAXIN2 (ATXN2) is differentially expressed during megakaryopoiesis. Here, we show that ATXN2 regulates protein homeostasis in megakaryocytic cells, which is needed to obtain functional platelets.

Atxn2-deficient mice contained normal platelet counts, but platelet aggregation was decreased upon activation of the αIIbβ3-integrin and Clec-2 receptor. Silencing ATXN2 in human megakaryocytic cells decreased overall protein synthesis and caused lower total protein content. ATXN2 associates with PABP and DDX6, proteins that control mRNA stability and translation. We subsequently profiled nontranslated subpolysomal RNA, and actively translated polysomal RNA following ATXN2 knockdown in megakaryocytic cells. ATXN2 mainly affected polysome recruitment of transcripts encoding proteins implicated in protein metabolism in general. Our results indicate that ATXN2 is involved in protein metabolism during megakaryopoiesis and is needed for proper platelet functionality.
Introduction

Megakaryopoiesis encompasses the differentiation of a hematopoietic stem cell towards mature megakaryocytes that subsequently undergo cytoskeletal remodelling, and ultimately release platelets into the bloodstream. Compared to many other differentiation processes, megakaryopoiesis contains several unique features, one of the most striking being endomitosis. During this incomplete cell division, the nuclear content of the cell is doubled without subsequent cytoplasmic separation. Incomplete division is repeated and as a result, mature megakaryocytes contain polylobulated nuclei up to 128N. It is believed that the increase in nuclear material allows for the accumulation of large amounts of mRNA. Many of the proteins synthesized from this increased mRNA pool are then packaged into cellular organelles called alpha granules. When mature megakaryocytes reorganize their cytoskeleton, alpha granules are actively transported and accumulate in the newly formed proplatelets. Ultimately, proplatelets are shed into the bloodstream where they circulate as mature, anucleate, platelets. Upon vascular damage, platelets interact with proteins of the subendothelial lining. This triggers signalling cascades, resulting in the activation and subsequent adhesion and spreading of platelets at the side of damage and the release of alpha granule contents.

To understand the complex mechanism of megakaryocyte differentiation including protein production and alpha granule synthesis, we performed gene expression profiling in human megakaryocytic cells. Among putative novel regulators of megakaryopoiesis we detected ATAXIN2 (ATXN2).

Recently, ATXN2 has also been found to play a role in hematological...
disorders. Genome-wide association studies revealed single nucleotide polymorphisms in the ATXN2 locus which associate with an increased risk for thrombotic antiphospholipid syndrome or autoimmune disease\textsuperscript{11-13}. ATXN2, encoded at the genomic locus SCA2\textsuperscript{14;15} is a 140 kDa protein that has mainly been studied as a polyglutamine repeat protein in the context of spinocerebellar ataxia type 2 (SCA2)\textsuperscript{16} and amyotrophic lateral sclerosis (ALS)\textsuperscript{13;17-19}. Considerable progress has been made deciphering the mechanism involved in aggregate formation of polyQ-expanded ATXN2 protein in neurodegenerative disease\textsuperscript{20}, but the function of physiological ATXN2 remains elusive. Several studies indicated that ATXN2 is involved in regulating mRNA translation. First, structural and functional analysis revealed domains involved in mRNA binding and translational regulation\textsuperscript{21;22}. Next, ATXN2 has been described to associate with stress granules\textsuperscript{23}, the rough endoplasmic reticulum\textsuperscript{24} and polyribosomes\textsuperscript{25}. Lastly, ATXN2 was reported to promote microRNA-mediated mRNA breakdown\textsuperscript{26;27}. In addition to a role in translation, ATXN2 may control receptor endocytosis, actin filament regulation and protein exocytosis\textsuperscript{28-31}. The observed effects may be due to regulation of translation of key factors in these processes; however this has not been investigated yet. Several studies used murine models to elucidate the function of Atxn2. Atxn2 deficient mice present with smaller litters, a segregation distortion with more male than female offspring and increased body weight\textsuperscript{32;33}. Brain phenotypes were subtle in Atxn2 knockout mice, suggesting that polyQ-expanded ATXN2 has stronger or converse effects than the loss of ATXN2.

We hypothesize that ATXN2 controls mRNA translation in megakaryocytes
during the accumulation of large amounts of transcripts encoding proteins required for platelet production. Mice lacking Atxn2 expressed normal numbers of platelets that were, however, defective in aggregation. Knockdown of ATXN2 in human megakaryopoietic cells reduced overall protein synthesis. Polysome profiling indicated that loss of ATXN2 alters translation of transcripts encoding proteins involved in protein synthesis and degradation. Thus, ATXN2 regulates general protein homeostasis in megakaryocytes, a process which is crucial for the production of fully functional platelets.

**Methods**

**Blood analysis**

Blood was drawn from Atxn2 knockout \((Atxn2^{-/-})\) and wildtype \((WT)\) animals by retro orbital puncture using heparin-coated glass capillaries (Hirschmann, Eberstadt, Germany), and collected in heparin-coated vials. Blood parameters were determined on a scil Vet abc Plus+ instrument (scil animal care, Oostelbeers, the Netherlands). Platelet rich plasma (PRP) was separated by centrifuging the blood 15 min at 50g.

**Mouse femur preparation and flow cytometry**

Mice were anaesthetised using Isofluran (Baxter, Unterschleissheim, Germany) and sacrificed by perfusion with 4% paraformaldehyde. Following perfusion, femurs were isolated, crushed and bone marrow was resuspended in PBS + 0.1% HSA. Bone marrow suspensions were then passed through a cell strainer (VWR, Amsterdam, the Netherlands) and counted on a Casy Cell Counter (Roche, Woerden, the Netherlands). Cells were then stained with
cMpl (Santa Cruz), Clec-2 (AbD Serotec, Puchenim, Germany), CD9-PE (Abcam), CD61-FITC, CD41-PE, KIT PerPCy5.5, (BD Biosciences); CD42a-FITC, CD42b-DL649, CD42c-FITC, CD42d-FITC (Emfret); CD31 PECy7 (Abcam) using a 1:1000 dilution for all antibodies. Flow cytometry based platelet aggregation assays were performed as previously described \(^{34}\). Receptor surface expression was measured using a flow cytometer (LSRII + HTS, BD Biosciences) and data was analyzed with FlowJo software version 9.2 (Tree Star Inc., Ashland, OR, USA).

**Human CD34\(^+\) cultures**

Peripheral blood stem cells were provided by the Sanquin Laboratory for Cell Therapy and obtained from leukapheresis material of healthy donors or donors in disease remission from Hodgkin lymphoma, multiple myeloma or breast cancer. For megakaryocyte cultures, CD34\(^+\) cells were cultured in CellGro medium (CellGenix, Frankfurt, Germany) supplemented with TPO (N-plate, Amgen, Breda, the Netherlands and IL-1\(\beta\) (PeproTech, Heerhugowaard, the Netherlands). At designated time points, cultures were either sorted for CD34 and CD41 expression, using the antibodies specified above, on an Aria II cell sorter (BD Biosciences). For phenotype analysis, cells were fixed with 1% PFA, washed with PBS + 0.05% BSA + 0.05M EDTA and incubated with CD34 PeCy7, CD41 APC or CD42b APC (all BD Biosciences). Samples were measured with flow cytometry (LSRII + HTS, BD Biosciences).

**Lentiviral knockdown constructs**

The lentiviral knock down vector SIN.PPT.CMV.GFP.U3Nhe1 was the kind gift
of N.A Kootstra (Academic Medical Center, Amsterdam, The Netherlands). We cloned a cassette containing the short hairpin RNA (shRNA) sequence under the control of a U6 promoter. The shRNA sequences used against ATXN2 were

sh93: GCCAAGACATATAGAGCAGTA, sh95: CCGAAGTGTGATTTGGTACTT and as a non-targeting shRNA control (shc002) CAACAAGATGAAGAGCACAA was used. All cloned short hairpins were verified by sequencing.

**Lentivirus production and lentiviral transduction**

Lentiviral particles were produced in 293T cells using the third generation system as described before \(^35\). Transductions were carried out as described before\(^35\). Cells were then seeded into megakaryocytic liquid cultures or used for colony formation assays. Megacult colony formation assay (Stem Cell Technologies, Grenoble, France) and Colony Gel colony formation assay (Cell Systems, Frankfurt, Germany) were carried out according to the manufacturer’s specifications. ClickIt total protein synthesis determination assay was performed according to the manufacturer’s specifications with an adjusted incubation time of one hour for AHA (LifeTechnologies).

**Immunoprecipitation and immunodetection**

Immunoprecipitation was carried out with CD34\(^+\)/CD41\(^+\) immature megakaryocytes. Cells were lysed in solubilization buffer (20mM Tris pH 8.0, 137mM NaCl, 2mM EDTA, 10% Glycerol, 1% NP-40), supplemented with protease inhibitor cocktail (Roche, Woerden, The Netherlands) and 25U/ml
benzonase (Merck, Darmstadt, Germany). 5µl of ATXN2 antibody (BD Biosciences) or IgG control antibody (Sanquin, Amsterdam, The Netherlands) was added to the lysate and incubated overnight at 4°C. The next day, sepharose beads (Pierce, Rockford, IL, USA) were added and incubated for at least 6 hours. Beads were then washed three times with solubilization buffer and eluted using SDS sample buffer. Samples were loaded on a 10% precast gel (Thermo Scientific, Waltham, MA, USA) and transferred onto nitrocellulose membranes using iBlot (Invitrogen, Bleiswijk, The Netherlands). Membranes were probed with ATXN2 (BD Biosciences), DDX6 (Novus Biologicals, Littleton, CO), PABP (Abcam, Cambridge, GB) and TIA-1 (Santa Cruz, Heidelberg, Germany). Secondary anti mouse-HRP antibody or anti rabbit-HRP antibody (both Dako, Glostrup, Denmark) was applied and membranes were developed by Enhanced Chemiluminescence (Pierce, Rockford, IL, USA).

Polysome profiling and microarray

Lysate of 7x10^6 Meg-01 cells was loaded on 7-46% sucrose gradients (10ml SW40 tubes UltraClear, Beckman, Woerden, The Netherlands). Gradients were spun at 35,000rpm for 3 hours (Beckman) and harvested as 17 fractions of 600µl. RNA was isolated as described 36. Fractions 1-8 were combined as subpolysomal RNA; fractions 9-17 as polysomal RNA. RNA was cleaned up using the Qiagen Micro kit according to the manufacturer’s protocol (Qiagen, Venlo, The Netherlands). Microarray was performed at Service XS (Leiden, The Netherlands). Samples were hybridised to the Illumina Human HT-12 v4 expression beadarray (Illumina, San Diego, CA). The data discussed in this
manuscript have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE63297.

R version 2.15.3 (www.r-project.org) and Bioconductor version 2.11 (www.bioconductor.org) were used for quality control and normalisation of the Illumina arrays. The Bioconductor package “lumi” is used for loading the Illumina data into R. VSN normalization is applied on the data using the 'lumiN' function. To maximize detection, no genes were excluded from the analysis. eBayes option was used to estimate the ‘average’ gene variability and to bring both high and low variability genes closer to the mean. The Benjamini Hochberg FDR values are calculated using the 'multtest' library (function: 'mt.rawp2adjp'). The q-values are calculated utilizing the 'qvalue' package. The Bioconductor 'lumiHumanAll.db' and the ‘GO.db' packages are used to get the most up-to-date annotations for the GO enrichment and other analysis. For the GO enrichment analyses, the calculated p-values are used together with the GO-annotations. Gene Set Enrichment Analysis (GSEA) (http://www.broadinstitute.org/gsea) is provided by the Broad Institute. The t-values are used to do a GSEAPreranked test to determine which biological pathways are significant in the underlying microarray experiment. The biological pathways are obtained from the MSigDB database.

De novo protein synthesis fluorescence label assay

Meg01 cells were transduced with short hairpins against ATXN2 or short hairpin controls and incubated for seven days. Subsequently, a ClickIT AHA Alexa Fluor 488 assay (Invitrogen) was performed according to the manufacturer’s specifications. Fluorescence was measured using flow
Results

Atxn2 deficiency causes a platelet aggregation defect

To investigate whether Atxn2 is required for megakaryopoiesis, or for hematopoiesis in general, we first investigated the blood cell composition in Atxn2 deficient (Atxn2\(^{-/-}\)) mice compared to wildtype (WT) animals. Platelet count, mean platelet volume as well as red and white blood cell counts were not significantly changed between Atxn2\(^{-/-}\) and WT animals (Figure 1A). To measure the efficiency of platelet aggregation, platelets from Atxn2\(^{-/-}\) and WT mice were activated with four specific agonists and microaggregation was measured by flow cytometry following a time course. Addition of botrocetin, which induces signaling via the GPIb (CD42b)/GPV(CD42d)/GPIX(CD42a) vWF receptor complex was comparable between Atxn2\(^{-/-}\) and WT platelets. Likewise, addition of collagen, which binds GPVI and integrin \(\alpha 2\beta 1\), was normal. Strikingly, aggregation of Atxn2\(^{-/-}\) platelets was significantly reduced compared to WT platelets upon addition of the \(\alpha llb\beta 3\) integrin (CD41, CD61) agonist PMA and the Clec-2 agonist Aggretin A (Figure 1B). Aggregation was not completely ablated in the Atxn2\(^{-/-}\) platelets compared to WT; moreover, it still followed the same kinetics. To investigate whether the decreased aggregation could be due to diminished receptor surface expression, we analyzed resting platelets from Atxn2\(^{-/-}\) and WT mice by flow cytometry for expression of the most abundant platelet receptors. Expression of all tested antigens was normal on Atxn2\(^{-/-}\) platelets compared to WT with the exception of a 30% decrease in CD31 (PECAM) (Figure 1B-C). Reduced receptor
Figure 1) ATXN2 deficiency causes a platelet aggregation defect. (A) Blood cell counts after retroorbital sampling from WT or Atxn2−/− mice, n=8 n.s.: not significant. (B) Aggregation of platelets from WT or Atxn2−/− mice, activated with PMA, Aggretin, botrocetin or collagen and incubated for designated times measuring percentage of aggregation over time, n=5, ** p<0.01. (C) Platelet receptor surface expression measured with flow cytometry on WT or Atxn2−/− platelets in resting state, n=5, * p<0.05
expression therefore does not explain the reduction in aggregation. These findings suggest that other factors might be involved to account for the reduced aggregation observed in Atxn2\textsuperscript{-/-} mice.

**Atxn2 deficiency does not alter megakaryocytic differentiation**

As platelets are derived from megakaryocytes, we next investigated at which stage Atxn2 expression influences megakaryocytic differentiation. Flushed femurs from WT and Atxn2\textsuperscript{-/-} animals yielded similar numbers of total bone marrow cells (Figure 2A). When bone marrow cell suspensions were analyzed for megakaryocytes using multicolour flow cytometry, the percentage of megakaryocytes (CD9\textsuperscript{+}/c-kit\textsuperscript{+}) was not significantly changed in Atxn\textsuperscript{-/-} mice compared to WT (Figure 2B). Furthermore, no differences in the expression of the megakaryocytic surface markers CD42b, CD61 were detected on bone marrow MK (Figure 2C). Also, when bone marrow megakaryocytes were analyzed for their differentiation state according to side scatter distribution and surface marker expression, no differences between WT and Atxn2\textsuperscript{-/-} mice were found (Supplemental Figure 1).

To address the impact of ATXN2 on megakaryopoiesis in more detail, we first determined the physiological expression of the ATXN2 protein in distinct stages of human megakaryocyte development: CD34\textsuperscript{+}/CD41\textsuperscript{-} hematopoietic stem and progenitor cells (HSPC), CD34\textsuperscript{+}/CD41\textsuperscript{+} immature megakaryocytes, and mature CD34\textsuperscript{-}/CD41\textsuperscript{+} megakaryocytes. ATXN2 expression increased from CD34\textsuperscript{+}/CD41\textsuperscript{-} to immature CD34\textsuperscript{+}/CD41\textsuperscript{+} megakaryocytes and sharply decreased again during differentiation to CD34\textsuperscript{-}/CD41\textsuperscript{+} megakaryocytes (Figure 2D). Next we depleted
Figure 2) ATXN2 deficiency does not alter megakaryocytic differentiation. (A) Bone marrow cellularity. Cell count was determined on bone marrow from WT or Atxn2-/- femurs, n=8. (B) Percentage of bone marrow megakaryocytes identified as CD9+/c-kit- cells found in total bone marrow cells. (C) Expression of megakaryocyte surface proteins from WT or Atxn2-/- mice. Flow cytometry was performed on cells isolated from femur, n=5. (D) Western Blot depicting ATXN2 expression during megakaryopoiesis. CD34+ cells derived from mobilized peripheral blood (MPB) were cultured in the presence of TPO and IL-1β and cell sorting was performed after 7 days of culture to obtain CD34+/CD41- and CD34+/CD41+ fractions. Subsequent
culture of the CD34+/CD41+ fraction resulted in a sample of terminally differentiated CD41+ cells. Immunodetection was carried out with ATXN2 and RhoGDI as a loading control. Representative image from n=3. (E) Lentiviral knockdown efficiency in CD34+ cells. CD34+ cells were transduced with shc002, sh93 or sh95. 48 hours after transduction cells were sorted for GFP expression and cultured for an additional three days before lysis. Immunodetection with ATXN2 and RhoGDI as loading control. Representative image from n=3. (F) Liquid megakaryocyte culture using CD34+ cells. Cells were transduced with sh93, sh95 or shc002 and cultured towards megakaryocytes in a liquid culture with TPO and IL-1β. Flow cytometry using monoclonal antibodies against CD34, CD41 and CD42b was used after 5 days of culture to assess differentiation. N=3, n.s.: not significant. (G) Megakaryocytic colony formation assay. CD34+ cells were transduced with shc002, sh93 or sh95 and seeded into semisolid medium promoting megakaryocytic colony formation. After two weeks of culture, CD41+ colonies were counted, shc002 set to 100%, n=3. (H) Colony formation assay to investigate hematopoietic lineage fate after ATXN2 knockdown. CD34+ cells were transduced with shc002, sh93 or sh95 and plated out single cell single well into semisolid medium. After two weeks, the amount of burst forming unit erythroid (BFU-E) and colony forming unit erythroid (CFU-E) as well as colony forming unit granulocyte macrophage (CFU-GM) and colony forming unit granulocyte, erythrocyte, macrophage (CFU-GEMM) were counted, n=3.

ATXN2 using lentiviral knockdown technology in CD34+ HSPC that were cultured towards the megakaryocytic lineage. Knockdown using two different short hairpins specific for ATXN2 (sh93 and sh95) almost completely abolished ATXN2 protein expression in CD34+ HSPC compared to short hairpin controls (shc002) (Figure 2E). Following 5 days of culture towards the megakaryocytic lineage, the distribution of CD34+ HSPC, CD34+/CD41+ immature megakaryocytes and CD41+ mature megakaryocytes was similar in ATXN2 knockdown cells transduced with either sh93 or sh95 compared to short hairpin controls (shc002) (Figure 2F). When transduced CD34+ cells were seeded into semisolid medium promoting the formation of megakaryocytic colonies, ATXN2 knockdown or shc002 gave rise to similar numbers of CD41+ megakaryocytic colonies (Figure 2G). We also examined the possible effect of ATXN2 knockdown on the commitment towards erythroid and granulocyte-monocyte progenitors, by plating single cell - single well
transduced CD34\(^+\) cells into conditioned semisolid medium. Compared to shc002, cells depleted for ATXN2 showed the same distribution of burst-forming unit erythroid (BFU-E), colony-forming unit erythroid (CFU-E), colony forming unit granulocyte macrophage (CFU-GM) and colony forming unit granulocyte, erythrocyte, macrophage (CFU-GEMM) (Figure 2H). Taken together, lack of ATXN2 does not influence hematopoietic lineage fate or early megakaryocytic differentiation in our human CD34\(^+\) in vitro system.

**ATXN2 associates with proteins involved in mRNA storage and decay**

The ATXN2 protein contains several domains facilitating binding to mRNA and other proteins (Figure 3A) \(^{21,23,25}\). Therefore, we investigated if interactions with known binding partners are also found in megakaryocytic cells, and whether ATXN2 may be associated with actively translated mRNA. The human megakaryoblastic cell line Meg-01 was used to separate mRNA in polyribosomal, translated, and subpolysomal, untranslated, RNA (Figure 3B). The RPL11 protein, which is part of the 60S ribosomal subunit, was prominently present in the subpolysomal fractions 5-7 of the gradient that contained the bulk of the total RNA as 60S ribosomal subunit plus 80S monoribosome (Figure 3B). Compared to RPL11, ATXN2 was only detectable in the first fractions of the gradient. These first fractions contain untranslated RNA and free proteins suggesting that most of ATXN2 is either not associated with RNA, or associates with untranslated RNA (Figure 3B). Immunoprecipitations demonstrated association of ATXN2 with poly(A) binding protein (PABP) and the RNA helicase DDX6 in immature primary human megakaryocytes (Figure 3C). The DDX6-PABP complex is known to
Figure 3. ATXN2 associates with proteins of mRNA storage and decay. (A) Schematic representation of functional domains within ATXN2. (B) Polysome profiling. Meg-01 cell lysate was separated by sucrose gradient centrifugation. Fractions were analysed for RNA concentration and immunodetection of ATXN2. (C) Immunoprecipitation using ATXN2 antibody in immature megakaryocytes. Membranes were probed for PABP, DDX6 and TIA-1. (D) ATXN2 protein content in Meg01 cells after lentiviral knockdown. A representative image is shown. (E) Light microscopic images of Meg01 cells after TPM1 knockdown. Scale bar 200 µm. (F) Total protein and total RNA content measured in Meg-01 cells after ATXN2 knockdown compared to shc002. (G) De novo protein synthesis was measured by flow cytometry as incorporation of a methionine analogue after transduction of Meg-01 cells with shATXN2 or SH002 expressing virus. n=5, ** p<0.01.

be involved in miRNA-mediated mRNA decay. mRNA targeted for degradation or translational silencing associates with GW182 (an RNAsase and marker for P-bodies) or TIA1 (an RNA-binding protein and marker for stress granules). Neither GW182 nor TIA1 associated with the ATXN2/PABP/DDX6 complex (Figure 3C). Taken together, our results show that ATXN2 associates with untranslated RNA and forms complexes with proteins involved in miRNA-mediated decay.
Given the association with untranslated mRNA and miRNA-mediated decay, we next investigated whether loss of ATXN2 impacts on protein synthesis. Meg-01 cells were transduced with shRNA constructs targeting ATXN2 (sh93 and sh95) or scrambled control (shc002) leading to decreased ATXN2 protein content in the cells (Figure 3D). Microscopically, ATXN2 knockdown did not alter the size or shape of Meg01 cells (Figure 3E). Compared to shc002, however, expression of sh93 or sh95 decreased total protein content by almost 50% whereas total RNA concentrations were not affected (Figure 3F). Incubation of transduced cells with a methionine analog to label newly synthesized protein revealed significantly lower methionine incorporation upon ATXN2 knockdown compared to shc002. Incorporation measured as mean fluorescence intensity was reduced by 50% with sh93 and by 70% with sh95 (Figure 3G). These results show that loss of ATXN2 results in decreased total protein content in megakaryocytic cells, caused by decreased de novo protein synthesis.

**ATXN2 controls the generation of proteins implicated in protein homeostasis**

The global reduction in protein synthesis suggests that translation of most transcripts is reduced. However, not all transcripts may be affected equally. To investigate which transcripts are under control of ATXN2, polyribosome profiling was performed on Meg-01 cells transduced with either shc002 or sh95 to analyze changes in the composition of untranslated and translated mRNA compared to shc002 (Figure 4A). Fractions 1-8 were pooled as subpolysomal mRNA, while fractions 9-17 were pooled as polysomal RNA
(Figure 4A). The RNA was hybridised to an Illumina beadchip array. To analyze global changes in protein expression, we focused our subsequent analysis on polysomal-bound mRNA and compared polysome-bound mRNA after ATXN2 knockdown with shc002. ANOVA analysis identified 172 transcripts differentially expressed in the polysomal fraction in three independent experiments. We calculated the fold change in expression between mRNAs in ATXN2 knockdown cells compared to cells transduced with shc002 for subpolysomal fractions as well and performed non-hierarchical, Euclidian clustering (Figure 4B-C, Supplemental Table 1). Due to poor hybridisation results, the subpolysomal fraction of shc002 and sh95 from one experiment was excluded from further analysis.

Clustering analysis based on the polysomal fractions revealed 9 clusters that we ordered in 6 functional groups (Figure 4C). Following ATXN2 knockdown, some transcripts were either up- or down-regulated in both polysomal and subpolysomal RNA (group C, n=37; group F, n=21). However, in group A (n=43) transcripts were increased in the polysomal fraction compared to subpolysomal expression and in group E (n=18) active translation in the polysomal fraction was reduced. Groups B and D contain transcripts that hardly changed in the subpolysomal fraction but were up- (B, n=44) or down-regulated (D, n=9) in polysomal mRNA suggesting a combination of transcriptional and translational regulation. Thus, ATXN2 knockdown altered the presence of specific transcripts in the polysomal, translated fraction compared to shc002. Gene set enrichment analysis showed that ATXN2 knockdown lead to a significant correlation with processes involved in mRNA turnover and translation, affecting processes as translation initiation ($p=10^{-12}$).
Figure 4. ATXN2 controls the generation of proteins implicated in protein homeostasis. (A) Polysome profiling after ATXN2 knockdown in Meg-01 cells. Meg-01 cells were transduced with shc002 or sh95, lysed, and spun down over a sucrose gradient. RNA concentrations of fractions were measured and samples for determining ATXN2 knockdown efficiency were taken. RNA from subpolysomal fraction 1-8 was pooled as was RNA from polysomal fraction 9-17. After pooling, RNA from pooled fractions was loaded on an Illumina Beadchip for expression profiling. (B) ErmineJ analysis of polysomal fractions from polysome profiling. (C) Heatmap showing fold increase (red) or decrease (green) of mRNAs after ATXN2 knockdown compared to shc002. Polysomal fractions (in blue) and subpolysomal fractions (in yellow) are shown after hierarchical clustering.
translation termination \((p = 10^{-12})\) and regulation of proteasomal ubiquitin-dependent protein catabolic process \((p = 10^{-12})\) (Supplemental Figure 2, Supplemental Table 2). Differentially expressed polysomal fractions also showed a significant positive correlation with both the mRNAs expressed in platelets\(^{38}\) and the Reactome translation pathway (Supplemental figure 2, Supplemental Table 2). However, when comparing the amount of shared RNAs, only 45 of the 172 selected transcripts were shared with the platelet enriched transcripts, whereas 153 of the 172 mRNAs were shared with the Reactome translation pathway. This indicates that ATXN2 knockdown impacts not just platelet specific transcripts but also general translation. Of note, the subpolysomal fraction showed a negative correlation to the platelet transcriptome\(^{38}\) and the Reactome translation pathway (Supplemental figure 2, Supplemental Table 2)

In conclusion, our results show that ATXN2 knockdown altered the composition of the translated, polysomal mRNA fraction in megakaryocytic cells thereby changing protein translation and degradation processes.

**Discussion**

Here, we analyzed how the RNA-binding protein ATXN2 impacts on protein homeostasis during megakaryopoiesis. ATXN2 is highly expressed in the first phase of megakaryopoiesis when cells become endomitotic and more translationally active. This suggests that ATXN2-dependent regulation of mRNA processing or translation may be involved in the generation of protein-dense, functional platelets. Mice lacking Atxn2 contained normal numbers of megakaryocytes and platelets. However, platelet aggregation in response to
specific agonists was reduced. Strikingly, the protein content of ATXN2 depleted megakaryocytes was almost 2-fold decreased, whereas mRNA levels were not significantly changed. The low protein content of ATXN2 depleted megakaryocytes was associated with a 50% reduction in protein synthesis in combination with increased translation of proteins involved in ubiquitination and protease degradation. Before platelet shedding, megakaryocytes accumulate proteins in alpha granules that are crucial for platelet function. Failure to accumulate these proteins may underlie the aggregation defect observed in mouse platelets.

Expression of all major platelet surface receptors was unchanged compared to WT which suggests that the reduced aggregation upon PMA and Aggretin A could be due to other factors. ATXN2 interacts with PABP and DDX6 in HEK 293T cells, SH-SY5Y cells and also in megakaryocytes. The same study also showed that in a yeast-2-hybrid screen, ATXN2 binds directly to DDX6 through its Lsm domain. While the presented data are convincing, the timing and functionality of this binding remain elusive. DDX6 participates in miRNA mediated decay and recruits the CCR4/NOT complex to mRNA to cause deadenylation, which subsequently leads to reduced translation and destabilisation of mRNA. However, DDX6 exerts its effect downstream of GW182 which has to bind to the target mRNAs first to induce miRNA mediated decay. GW182 binds to the PAM2 motif of PABP, occupying the same structure that also ATXN2 binds to. This concurrence for the PAM2 motif means that binding of both GW182 and ATXN2 to PABP is impossible, reflected in our findings that upon immunoprecipitation with ATXN2, GW182
could not be co-precipitated. However, since ATXN2 is outcompeted by GW182 to induce RNA decay, the question remains why binding between ATXN2 and DDX6 occurs during the progression of mRNA decay. Direct or indirect interaction of ATXN2 with PABP and DDX6 may either enhance or inhibit the recruitment of CCR4/NOT by DDX6.

Recently, ATXN2 was shown to bind to AU-rich elements thereby promoting RNA stability and translation \(^{22}\). We also analyzed the occurrence of AU-rich elements in the various clusters observed in the microarray but could not detect any correlation. Still, the interaction of ATXN2 with DDX6 may suggest protection from RNA decay by DDX6. We found that protein synthesis was reduced upon loss of ATXN2, which also suggests a positive role of ATXN2 in translation regulation. The unresolved question is whether ATXN2 binds PABP/DDX6 in presence or absence of mRNA. It is currently unknown if ATXN2 sequesters PABP/DDX6 and prevents mRNA binding, or whether it binds mRNA and thereby inhibits PABP/DDX6 function on the same transcript.

Amongst the transcripts that we found increased in the polysomal, translated fraction after ATXN2 knockdown were members of the ubiquitination pathway such as the E3 ubiquitin ligase UBEC3 and the F-box protein FBXO18. During megakaryopoiesis, proteins and specific RNAs are actively transported into the developing platelets \(^{40,41}\). In a recent RNA sequencing study of human platelets, several RNAs coding for ubiquitination proteins were detected, including UBEC3 and FBXO18 \(^{42}\). Taking into consideration that platelets are capable of de novo protein synthesis and contain functional proteasomes \(^{43}\) it
is therefore possible that the impaired aggregation observed in Atxn2−/− mice is caused by increased ubiquitin-mediated protein degradation. This hypothesis is reinforced by observations that integrin αIIbβ3 (CD41/CD61) has been shown to directly bind an E3 ubiquitin ligase and that protein ubiquitination takes place in activated platelets 44, placing protein decay directly downstream of the integrin signalling that was decreased in Atxn2−/− mice.

In a recent publication, Yokoshi and colleagues show that ATXN2 binds to AU-rich elements thereby promoting RNA stability and translation 22. Using microarray analysis we identified transcripts of which translation was reduced upon ATXN2 knockdown which indeed could indicate faster mRNA decay, but most transcripts showed increased translation upon ATXN2 knockdown. This seems contradictory with the conclusion that ATXN2 stimulates mRNA translation. It is therefore also possible that the transcripts with increased polysome recruitment upon ATXN2 knockdown are not those regulated by ATXN2, but are positively controlled by mechanisms that remain intact, or even increase upon loss of ATXN2.

Our results clearly demonstrate a role for ATXN2 during megakaryopoiesis and provide the first evidence for the importance of proper protein accumulation for platelet reactivity. Still, there seems to be a discrepancy between the physiological role of ATXN2 and the pathogenicity caused by mutant polyglutamine ATXN2. It was assumed that the clinical manifestations of neurodegenerative diseases are caused by the accumulation of the mutant protein in the cytoplasm of affected neurons, rendering the protein
dysfunctional, a state comparable to a loss of function in mice with ablation of Atxn2. However, two independent ATXN2 knock out mice strains have been generated, both of which show diverse, but rather subtle, phenotypes\textsuperscript{32,33}. It is especially striking that even though mutant ATXN2 was discovered as a protein causing neurodegenerative disease, loss of the physiological protein does not cause severe effects in murine brain\textsuperscript{33}. Recent studies showed that mutant ATXN2 does not accumulate by itself, but in complexes containing PABP\textsuperscript{20}. Combined with our results showing that loss of ATXN2 results in declined de novo protein synthesis and decreased total cellular protein content, a strong relationship of ATXN2 with protein homeostasis emerges. It is therefore possible that degeneration of affected neurons in SCA2 patients is the result of deregulated protein synthesis caused by the accumulation of PABP-ATXN2 complexes. This aggregation would eventually deprive the cells of essential proteins, leading to impaired neuron functionality. If indeed this hypothesis can be confirmed is beyond the scope of this manuscript but the contribution of ATXN2 to regulation of protein homeostasis may provide new leads to unravel the mechanism behind SCA-2.
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Authorship contributions and disclosures

SZ, MM, SP, FdS, ED and MH performed experiments, SZ and DCT designed and analyzed experiments and wrote the paper, GA revised the manuscript and provided materials, LG and MM analyzed data and revised the manuscript, MvL analyzed experiments and revised the manuscript. The authors declare no competing financial interests.

Abbreviations List

ATXN2: ATAXIN-2
CLEC-2: C-type lectin-like receptor 2
WT: Wildtype mice
BFU-E: burst-forming unit erythroid
CFU-E: colony-forming unit erythroid
CFU-GM: colony-forming unit granulocyte macrophage
CFU-GEMM: colony forming unit granulocyte, erythrocyte, macrophage
HSPC: hematopoietic stem and progenitor cell
TIA-1: T-cell restricted intracellular antigen-1
DDX6: DEAD-box helicase 6
PABP: poly(A)-binding protein C1
PMA: phorbol myristate acetate
Reference List


32. Kiehl TR, Nechiporuk A, Figueroa KP et al. Generation and


Supplemental methods

Antibodies and reagents
Antibodies used were CD61-FITC, CD41-PE, KIT PerCPCy5.5, (BD Biosciences); CD42a-FITC, CD42b-DL649, CD42c-FITC, CD42d-FITC (Emfret, Eibelstadt, Germany); CD31 PECy7, CD9-APC, CD9-PE (Abcam), cMpl (Santa Cruz), Clec-2 (AbD Serotec, Pucheim, Germany) in a 1:1000 dilution for all antibodies. For platelet aggregation we used phorbol myristate acetate (PMA 100ng/ml, Sigma-Aldrich, Zwijndrecht, the Netherlands), Botrocetin (10µg/mL, Sigma-Aldrich), collagen (10µg/mL), convulxin (0.5µg/ml, Santa Cruz, Heidelberg, Germany) or Aggretin A (30 nM, a kind gift of Prof. Dr. Johannes A. Eble).

Human CD34+ cells and culture systems
Donors and patients had been treated with chemotherapy and G-CSF (5-10 µg/kg/day) and signed informed consent for the use of leftover material for research purposes (METCnr. 04/042, #04.17.370, AMC, Amsterdam, the Netherlands). CD34+ cells were isolated within 48 hours after arrival of the material using magnetic cell sorting (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer’s specifications. Purity of the isolated cells was determined by flow cytometry with mouse monoclonal antibodies raised against human CD34 or CD41 (BD Biosciences). All samples displayed a purity of higher than 90 % CD34 and less than 5% CD41.
Supplemental Figure 1. Differentiation status of bone marrow megakaryocytes. Cell suspensions from either WT or Atxn2−/− femur were gated on all nucleated cells and divided into immature ckit+/SSClow and more mature ckit+/SSChigh populations. Immature or mature populations were further subdivided according to surface marker expression based on CD31, CD61 or CD42b.
Supplemental Figure 2. Gene set enrichment analysis. Expression profiling from subpolysomal and polysomal fractions is compared with the Broad Institute data set.

Supplemental Table 1. Fold change of all transcripts in subpolysomal (1-8) or polysomal fractions (9-17) after ATXN2 knockdown compared to shc002. Fold changes are displayed to compensate for differences between independent experiments.

Supplemental Table 2. Overview of genes found in gene set enrichment analysis. Listed genes are found when comparing subpolysomal or polysomal gene expression after ATXN2 knockdown compared to either the platelet transcriptome as analyzed by Gnatenko et al or the Reactome Translation pathway.