# Impaired ribosome function and the molecular biology of the 5q- syndrome

The following presentation has been modified from its original version. It has been formatted to fit this screen and edited for content, to run in the time allotted

Leukaemia & Lymphoma Research
BEATING BLOOD CANCERS

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**WHO Classification and Criteria for the Myelodysplastic Syndromes.***

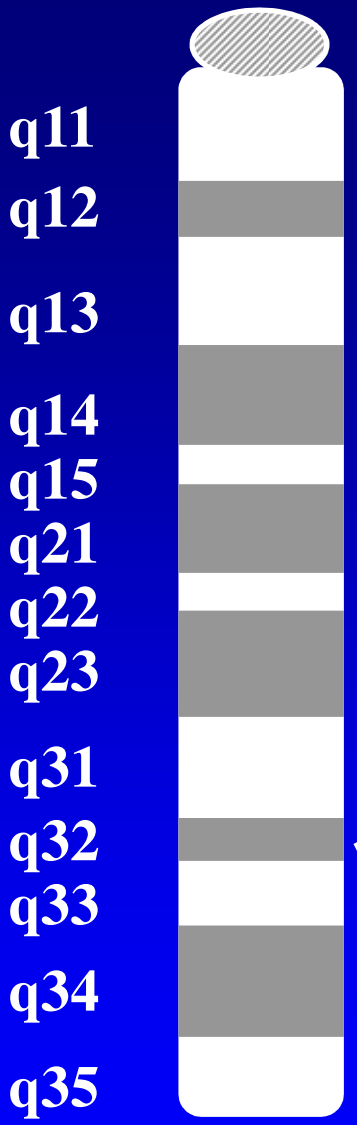
| Disease | Blood Findings | Bone Marrow Findings |
|---|---|---|
| Refractory anemia | Anemia, no or rare blasts | Erythroid dysplasia alone, <5% blasts, <15% ringed sideroblasts |
| Refractory anemia with ringed sideroblasts | Anemia, no blasts | Erythroid dysplasia alone, <5% blasts, ≥15% ringed sideroblasts |
| Refractory cytopenia with multilineage dysplasia | Cytopenias (bicytopenia or pancytopenia), no or rare blasts, no Auer rods, <1 billion monocytes per liter | Dysplasia in ≥10% of cells in ≥2 myeloid cell lines, <5% blasts, no Auer rods, <15% ringed sideroblasts |
| Refractory cytopenia with multilineage dysplasia and ringed sideroblasts | Cytopenias (bicytopenia or pancytopenia), no or rare blasts, no Auer rods, <1 billion monocytes per liter | Dysplasia in ≥10% of cells in ≥2 myeloid cell lines, <5% blasts, no Auer rods, ≥15% ringed sideroblasts |
| Refractory anemia with excess blasts, type 1 | Cytopenias, <5% blasts, no Auer rods, <1 billion monocytes per liter | Unilineage or multilineage dysplasia, 5–9% blasts, no Auer rods |
| Refractory anemia with excess blasts, type 2 | Cytopenias, 5–19% blasts, occasional Auer rods, <1 billion monocytes per liter | Unilineage or multilineage dysplasia, 10–19% blasts, occasional Auer rods |
| Myelodysplastic syndrome, unclassified | Cytopenias, no or rare blasts, no Auer rods | Unilineage dysplasia in granulocytes or megakaryocytes, <5% blasts, no Auer rods |
| Myelodysplastic syndrome associated with isolated del(5q) | Anemia, <5% blasts, platelet count normal to increased | Normal-to-increased megakaryocytes with hypolobated nuclei, <5% blasts, no Auer rods, isolated del(5q) |

* Information is from Vardiman et al.[2]

# Ribosome dysfunction in the 5q- syndrome

- **Introduction and mapping of the CDR**
- **Role of haploinsufficiency of RPS14 and p53 activation in molecular pathogenesis**
- **Defective translation and treatment with L-leucine**
- **Cooperating genetic events in disease pathogenesis**

# Commonly deleted regions (CDR) of 5q in MDS/AML




CSF2/IL3
TCF1B
IL9
SMAD5
D5S393
D5S89
D5S399
SPOCK
D5S479
AFMB350YB1
D5S1983
D5S414
EGR1
D5S500
D5S476
D5S1372
D5S594

ADRB2
CSF1R
RPS14
SPARC
MEGF1
GLRA1

**MDS/AML and t-MDS/t-AML**

Zhao et al, Genomics 1997

1- 1.5 Mb
5q31

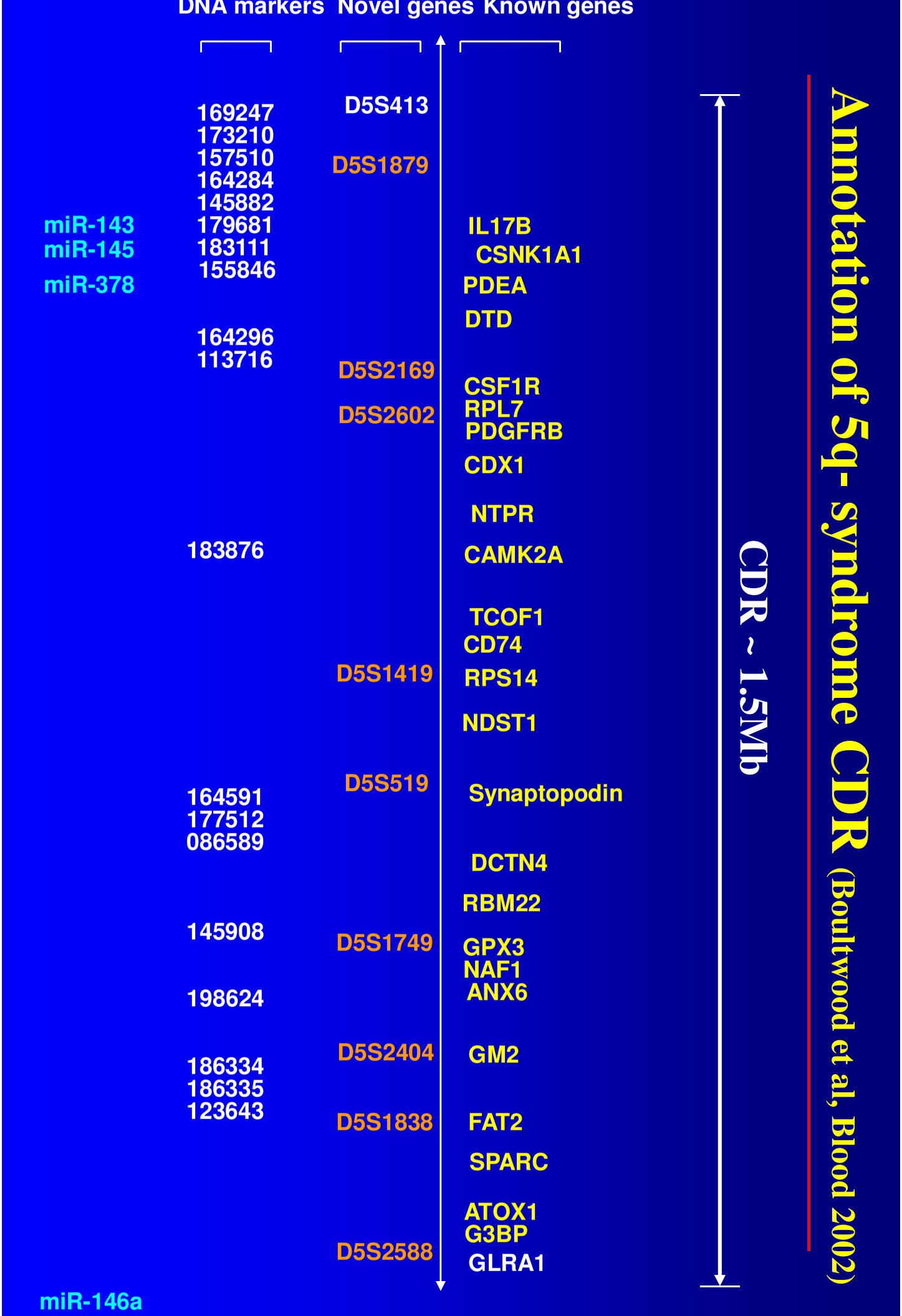
**5q- syndrome**

Boultwood et al, Blood 2002

~1.5 Mb
5q32

# Mapping the commonly deleted region (CDR) of 5q- syndrome

- performed detailed FISH/molecular mapping of del(5q) bps in group of patients with the 5q- syndrome using panel genes/DNA markers localised 5q31-q34 and identified CDR (Boultwood *et al*, 1994; Jaju *et al*, 1998)
- narrowed CDR 1.5 Mb region at 5q32-q33 flanked by D5S413 and GLRA1 (Boultwood *et al*, Blood 2002)
- confirmed localisation of 5q- syndrome CDR using array CGH and SNP array analysis (Wang *et al*, Haematologica 2008)


DNA markers
Novel genes
Known genes
169247
173210
157510
164284
145882
179681
183111
155846
164296
113716
183876
164591
177512
086589
145908
198624
186334
186335
123643
D5S413
D5S1879
D5S2169
D5S2602
D5S1419
D5S519
D5S1749
D5S2404
D5S1838
D5S2588
IL17B
CSNK1A1
PDEA
DTD
CSF1R
RPL7
PDGFRB
CDX1
NTPR
CAMK2A
TCOF1
CD74
RPS14
NDST1
Synaptopodin
DCTN4
RBM22
GPX3
NAF1
ANX6
GM2
FAT2
SPARC
ATOX1
G3BP
GLRA1
miR-143
miR-145
miR-378
miR-146a
CDR ~ 1.5Mb
Annotation of 5q- syndrome CDR (Boultwood et al, Blood 202)

# Ribosome dysfunction in the 5q- syndrome

- Introduction and mapping of the CDR
- Role of haploinsufficiency of RPS14 and p53 activation in molecular pathogenesis
- Defective translation and treatment with L-leucine
- Cooperating genetic events in disease pathogenesis

# Mutation and expression analysis of genes mapping to CDR in 5q- syndrome

- mutation analysis performed on exons of 40 genes mapping to CDR in 12 patients with 5q- syndrome -no mutations identified

- data supports haploinsufficiency model (gene dosage effect resulting from loss single allele of a gene)

- majority of genes in CDR show expression levels consistent with loss of a single allele in CD34+ cells of 5q- syndrome patients

  - noted that ribosomal gene RPS14 (candidate by analogy with DBA), showed haploinsufficiency in 5q- syndrome

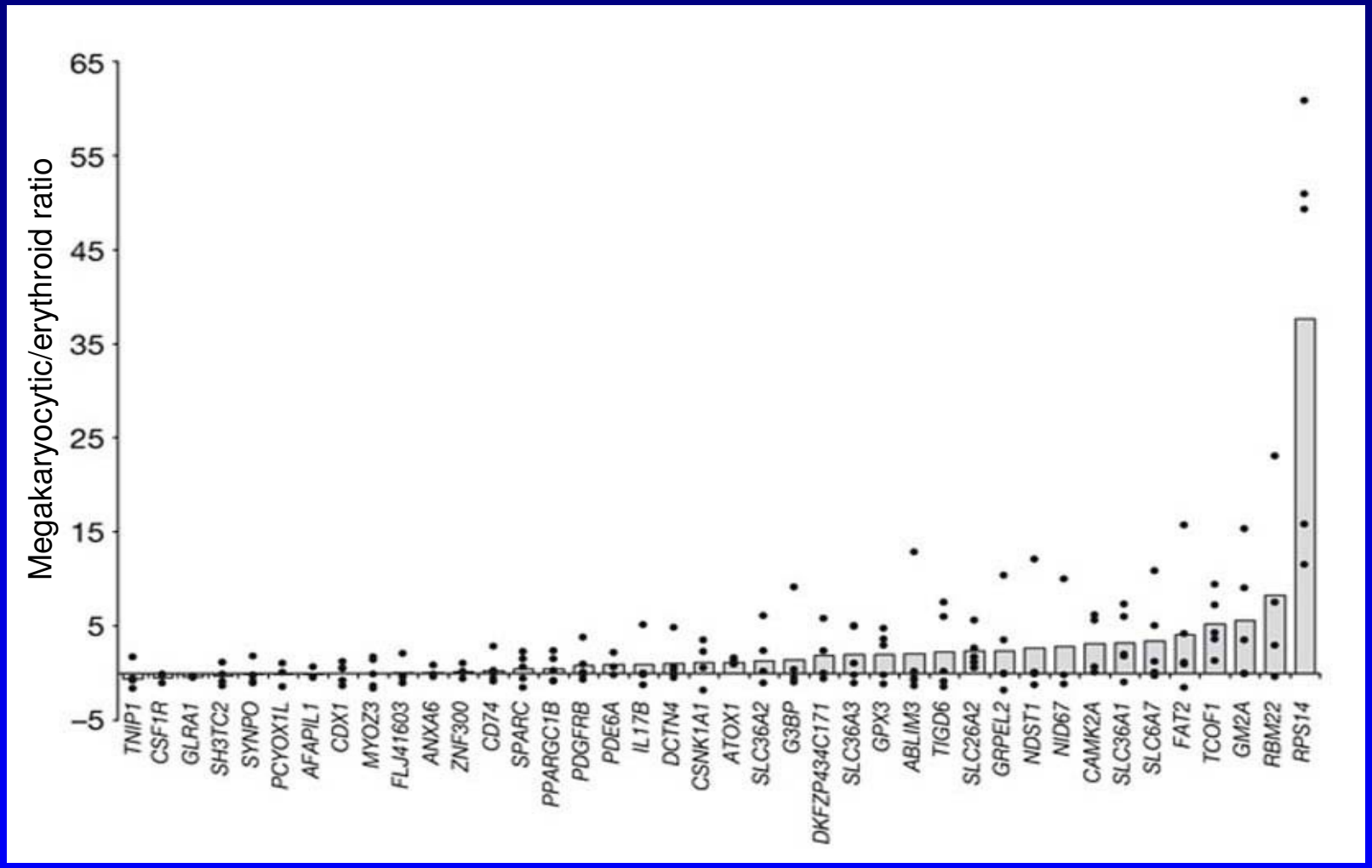
Boultwood *et al*, BJH 2007

# Identification of RPS14 as a 5q- syndrome gene: screen of the CDR

- used an RNAi- based approach to knockdown expression of 40 genes mapping to the CDR of 5q- syndrome in CD34+ cells
- reduced expression of RPS14 in CD34+ cells caused decrease in production of erythroid cells with relative preservation of megakaryocyte cells
- haploinsufficiency of RPS14 causes a block in production of terminally differentiated erythroid cells

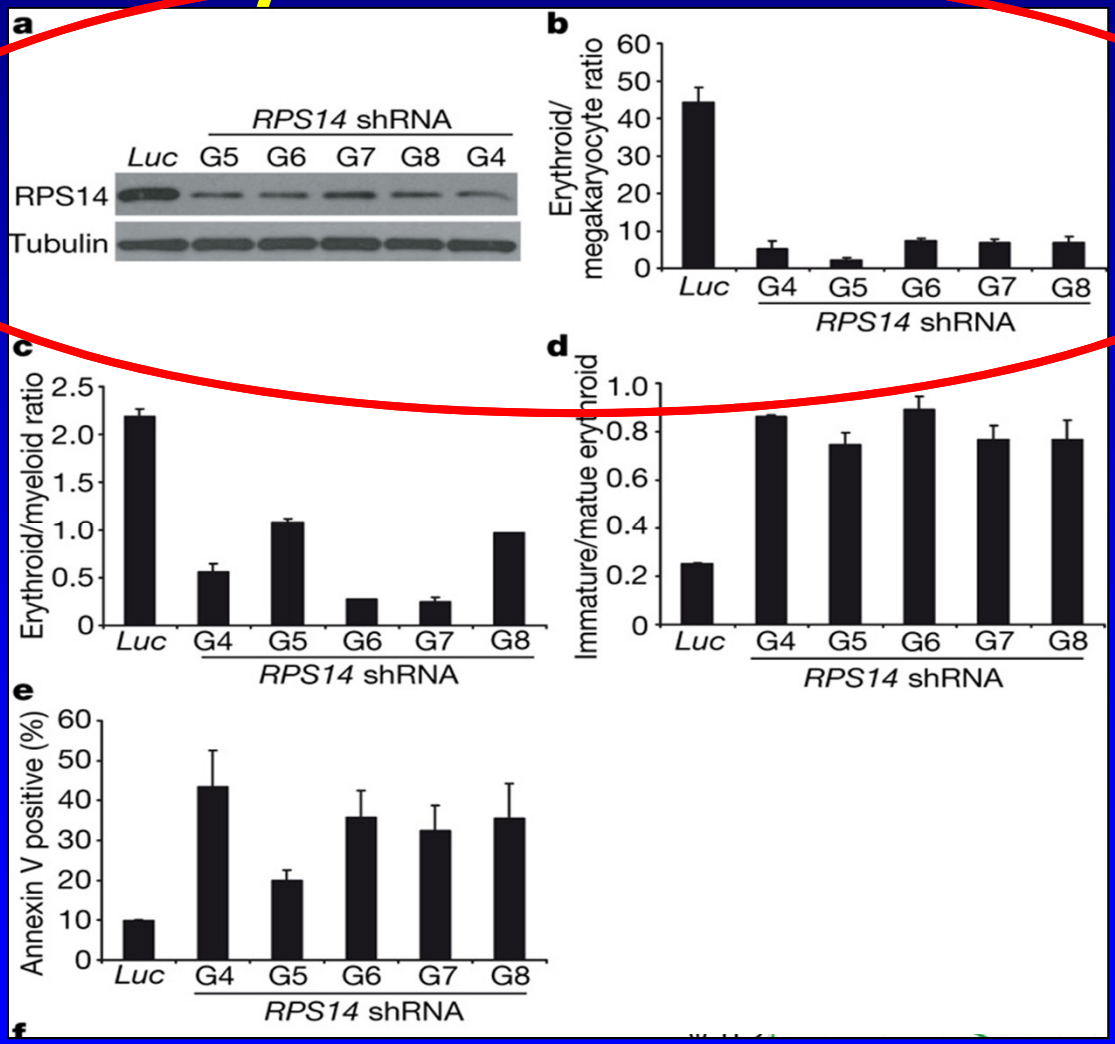
**B. Ebert *et al*, Nature 2008**

# Screen of the common deleted region for the 5q- syndrome




- **Knockdown of all of the genes mapping to the CDR of the 5q- syndrome in CD34+ cells using an RNAi - based approach (lentivirally expressed shRNAs)**
- ***RPS14* knockdown decreased erythroid differentiation relative to megakaryocytic differentiation**

# RPS14 knock down impairs erythroid differentiation




B. Ebert et al Nature 2008

# Identification of RPS14 as a 5q- syndrome gene: rescue of the erythroid defect

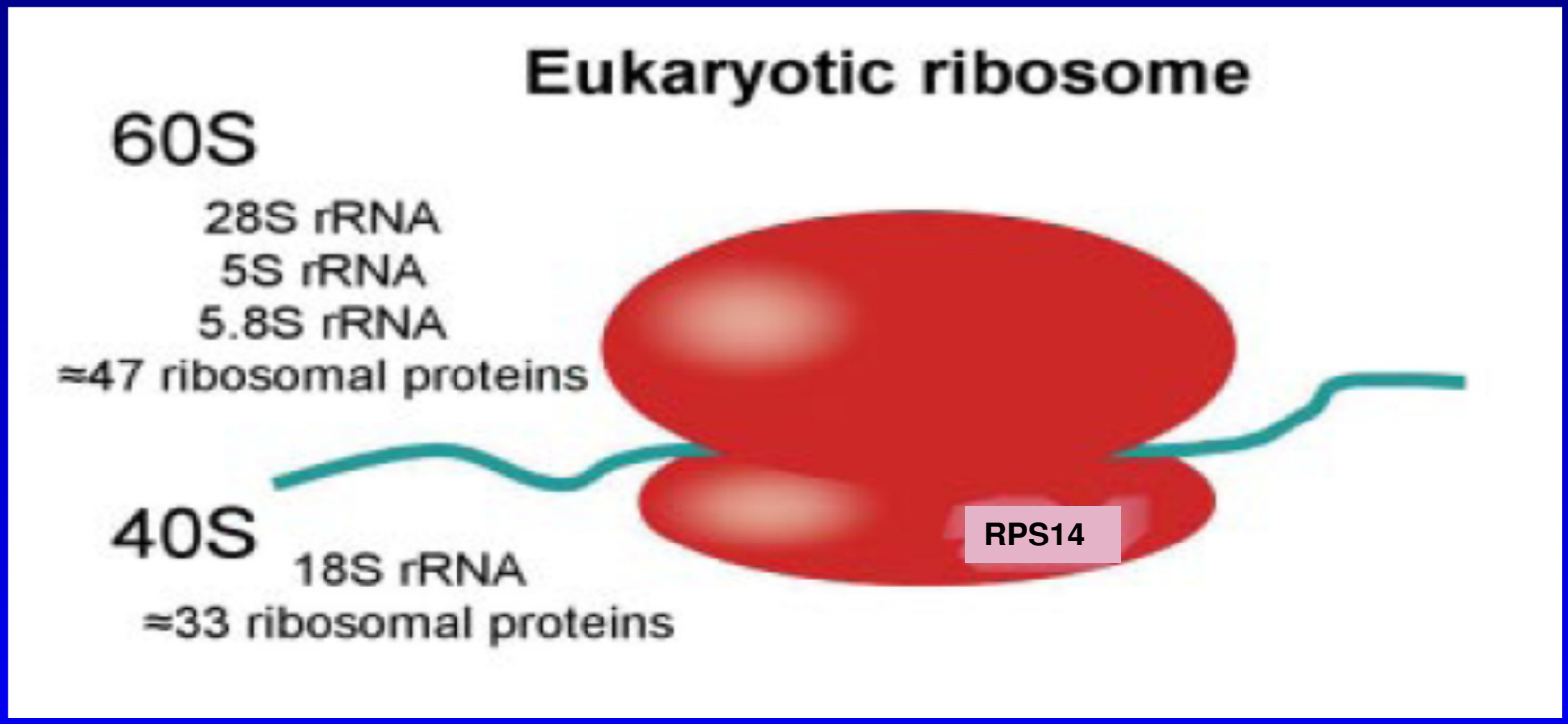
- increased RPS14 expression in CD34+ cells from patients with 5q- syndrome
- Found increased erythroid differentiation in 5q- patients but not in MDS patients without del(5q), by FACS analysis
- normalisation of RPS14 expression level in 5q- syndrome rescued the erythroid differentiation defect – suggesting that RPS14 plays a critical role in erythroid defect in the 5q- syndrome

B. Ebert *et al*, Nature 2008

# Ribosomes-background

- the ribosome is responsible for translating the genetic code into polypeptides
- mammalian ribosome is divided into a small (40S) ribosomal subunit and a large (60S) subunit, each composed of rRNA and ribosomal proteins
- ribosomal proteins are required for production of ribosome assembly intermediates and mature ribosomes

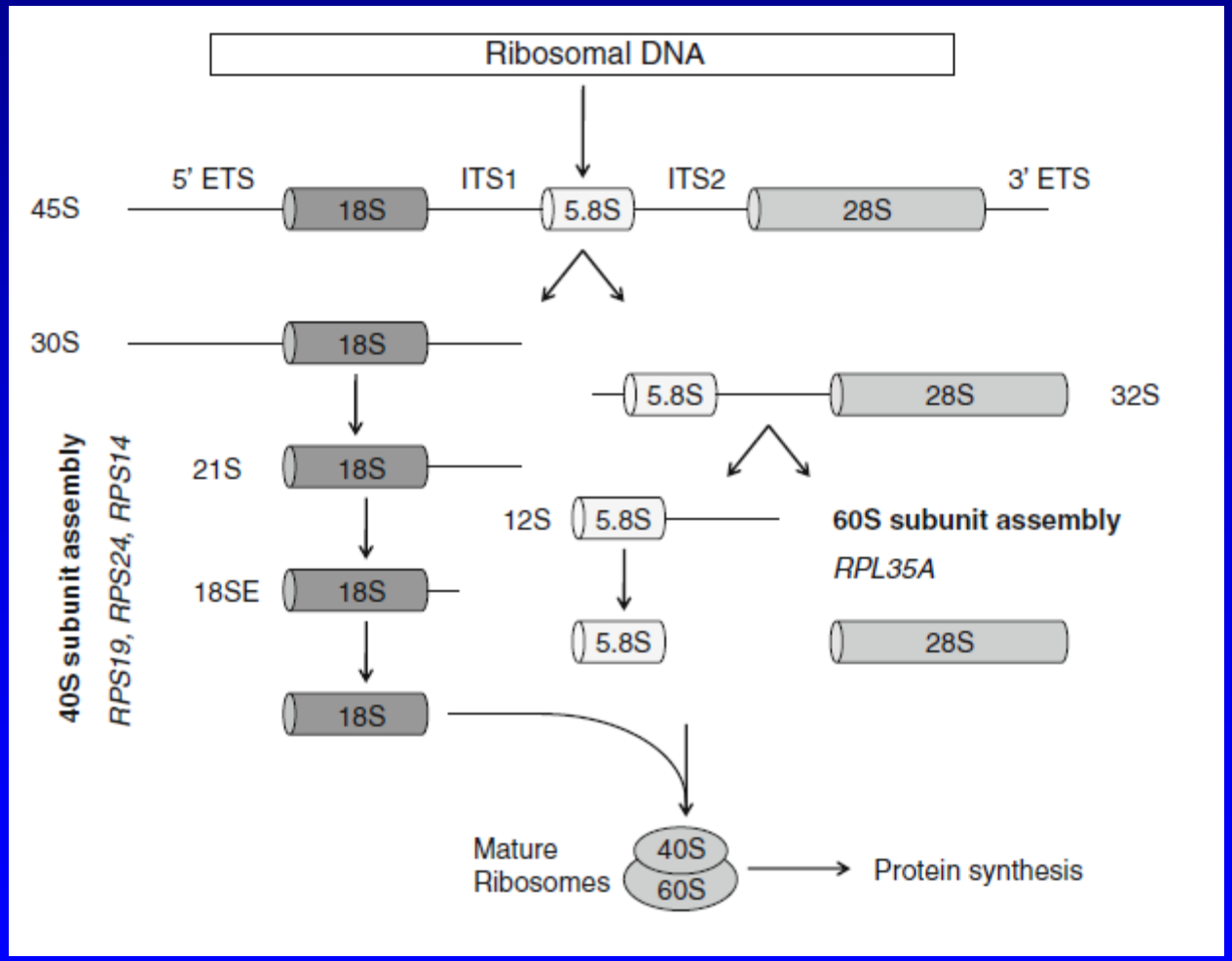
# The ribosome and RPS14

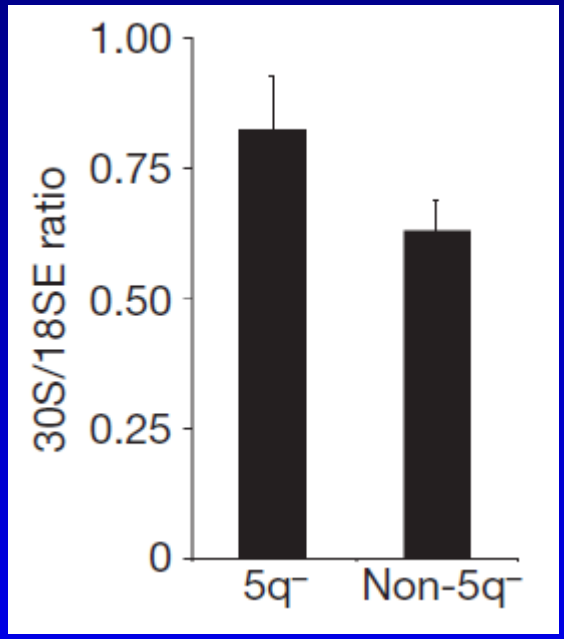



- component of 40S ribosomal subunit
- involved in the maturation of the 40S ribosomal subunit

# Pre-rRNA processing defect occurs in 5q- syndrome

## Eukaryotic ribosome biogenesis




**30S/18SE pre-rRNA ratio is increased in 5q- syndrome bone marrow RNA compared to MDS without 5q - Northern blots**

**B. Ebert *et al*, Nature 2008**

**RPS14 involved in processing of 18S pre-ribosomal RNA**

# 5q- syndrome analogy with DBA

- **Diamond-Blackfan anaemia is a congenital hypoplastic anaemia**

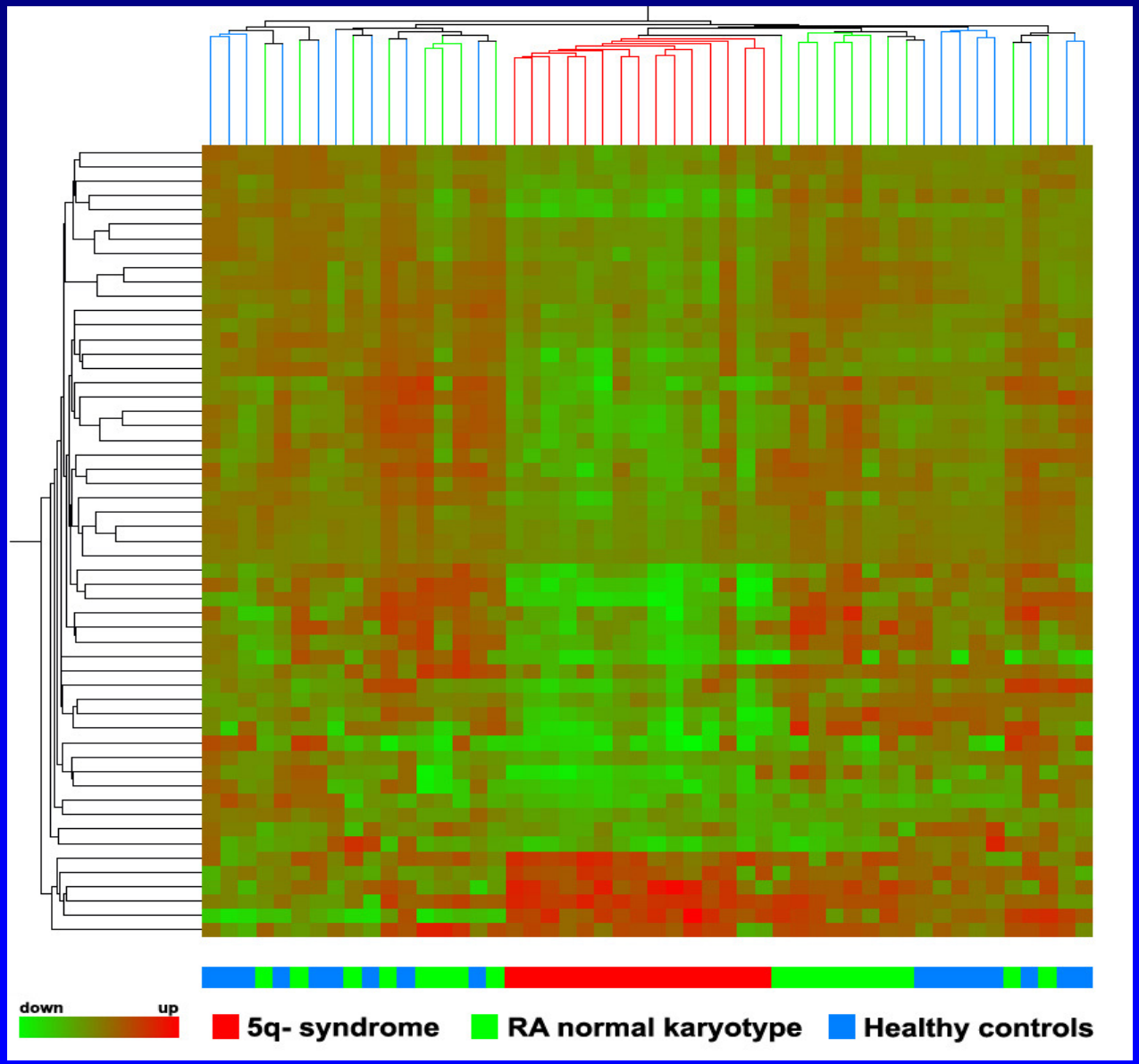
- **25% patients with Diamond-Blackfan anaemia show haploinsufficiency (via gene mutation) of the closely related ribosomal protein RPS19, also required for the maturation of 40S ribosomal subunits (Draptchinskaia *et al*, 1999)**

# RPS14 haploinsufficiency associated with deregulation of multiple ribosomal genes in 5q- syndrome

- CD34+ cells of DBA patients show deregulation of multiple ribosomal and translation related genes
- using GEP we have shown that 55 of 579 ribosomal and translation related genes were significantly differentially expressed in CD34+ cells of 15 5q- syndrome patients as compared to 18 RA with a normal karyotype and 17 healthy controls, e.g. RPS23, EIF2A
- suggests that 5q- syndrome represents a disorder of aberrant ribosomal biogenesis

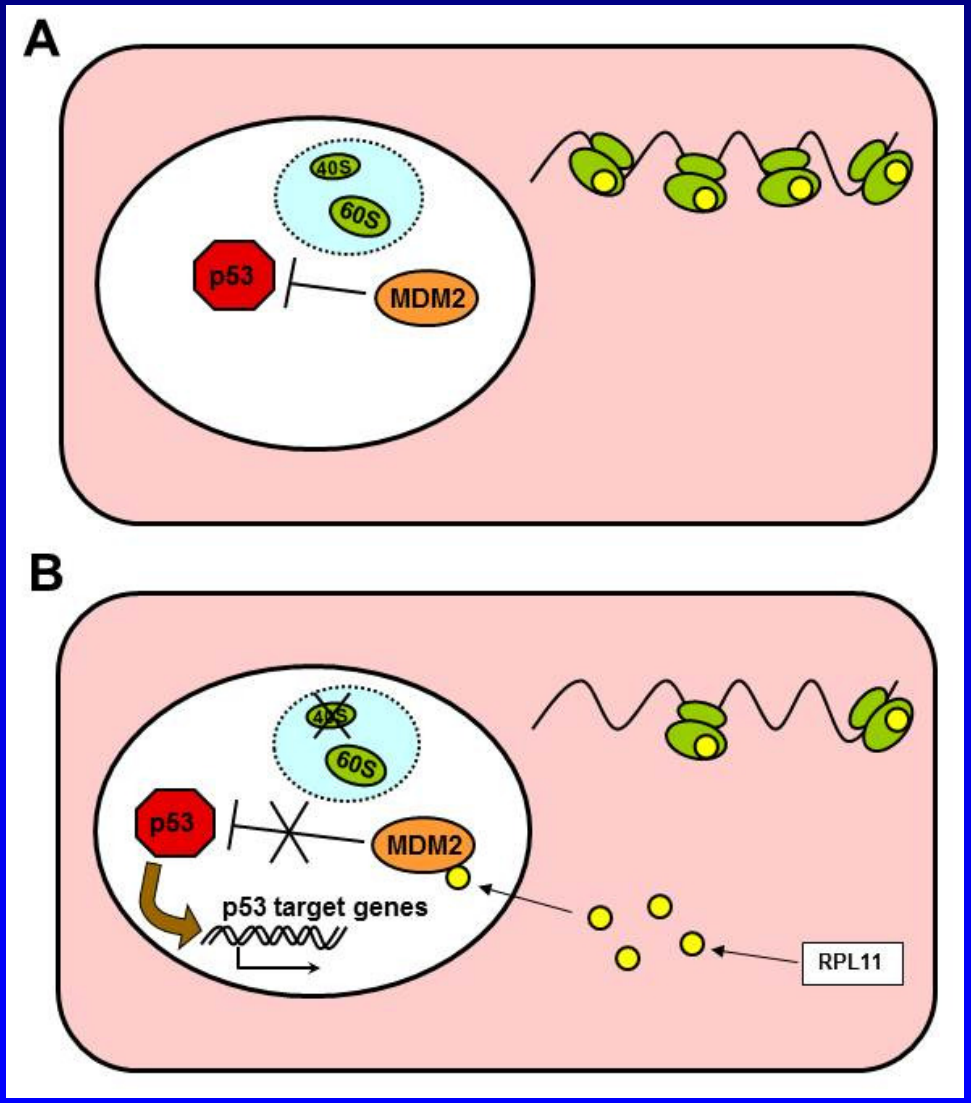
Pellagatti et al, BJH 2008

# Hierarchical clustering of 55 differentially expressed ribosomal and translation genes in MDS




- 5q- syndrome patients separated from other RA patients and controls solely on basis of deregulated expression of these ribosomal genes

# Ribosomal haploinsufficiency causes p53 activation

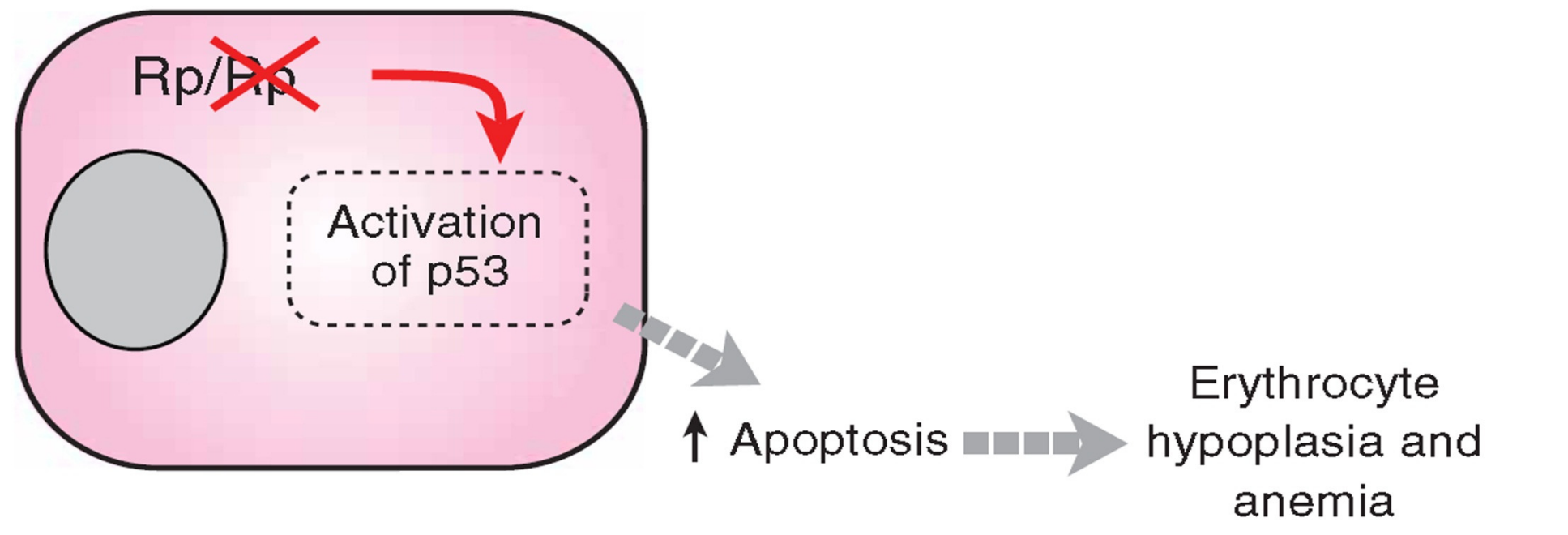



Normal cell in unstressed conditions, with unperturbed ribosome biogenesis and steady levels of p53

Ribosomal haploinsufficiency leads to up-regulation of RPL11, which binds to MDM2 inhibiting ubiquitylation and degradation causing p53 activation and target gene activation resulting in apoptosis and cell-cycle arrest

# Ribosome protein haploinsufficiency, p53 activation and DBA

- **mutations in Rps19 cause dark skin, reduced body size and reduced erythrocyte count in mouse through activation of p53 and target genes and inhibition of p53 alleviates the phenotype (McGowan *et al*, Nat Genet 2008)**



# Generation of a mouse model of 5q- MDS

**Dr Andrew McKenzie, LMB, Cambridge**

- **used cre-*lox*P-mediated chromosomal engineering to generate a mouse model of the 5q- syndrome**
- **showed that segmental haploidy of the Cd74-Nid67 interval, syntenic with a region contained within the 5q- syndrome CDR (containing Rps14), results in the key features of the human disease:**

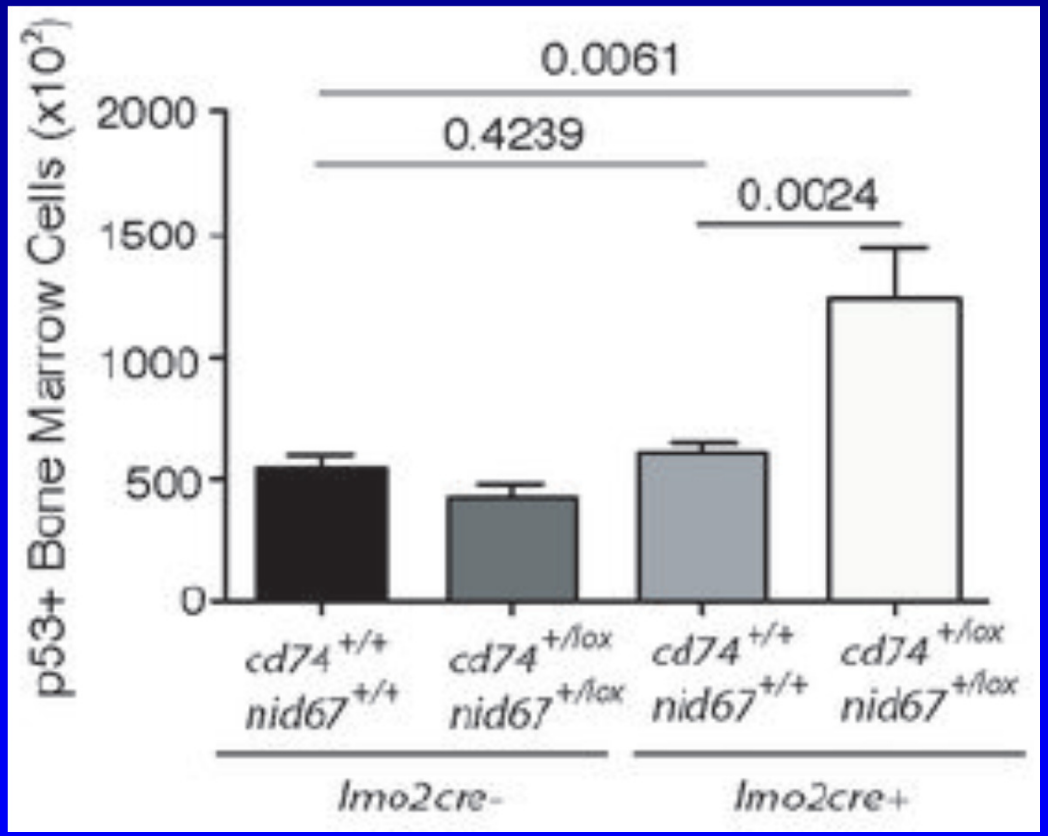
  **- macrocytic anaemia**

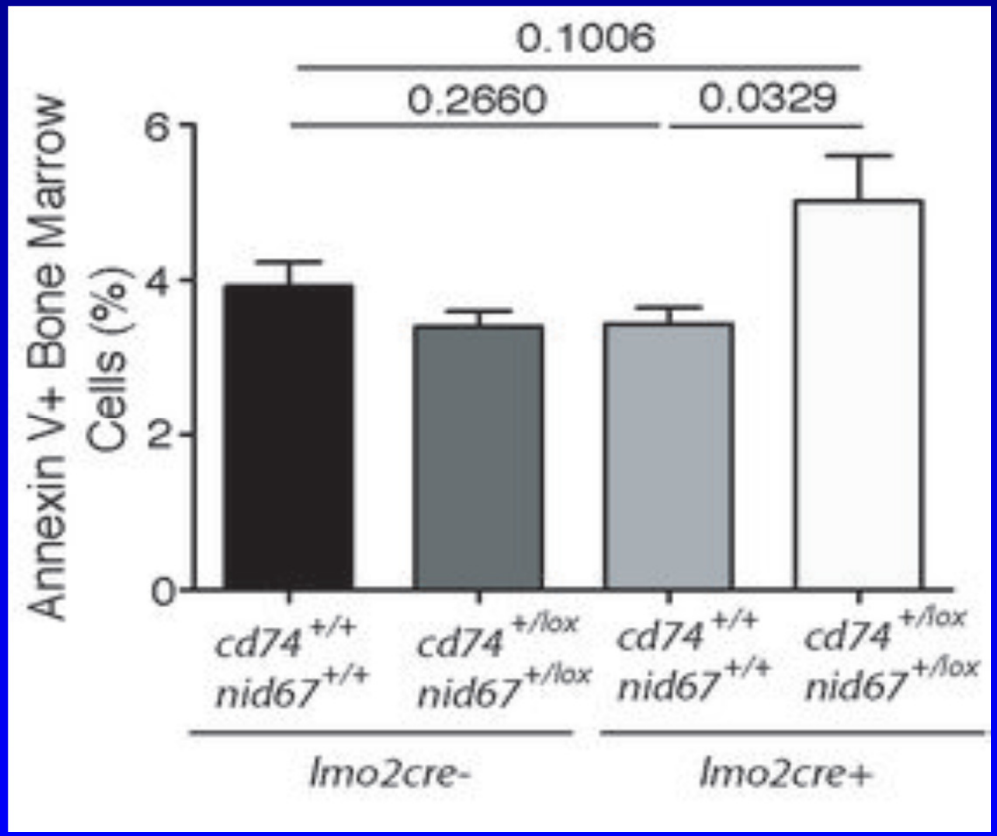
  **- prominent erythroid dysplasia**

  **- monolobulated megakaryocytes in the bone marrow**

**Barlow et al, Nature Medicine, 2010**

# High expression of p53 and increased apoptosis in bone marrow of Cd74-Nid67 deletion mouse




**a) numbers of p53+ bm cells**

**b) Annexin V staining bm cells**

# p53 and the 5q- syndrome mouse model

- the Cd74-Nid67 deletion mice were crossed with $p53^{-/-}$ mice - completely rescued the progenitor cell defect; restoring CMP, MEP, GMP, and HSC bone marrow populations
- suggests that a p53-dependent mechanism underlies the pathophysiology of the 5q- syndrome

Barlow et al, Nature Medicine, 2010

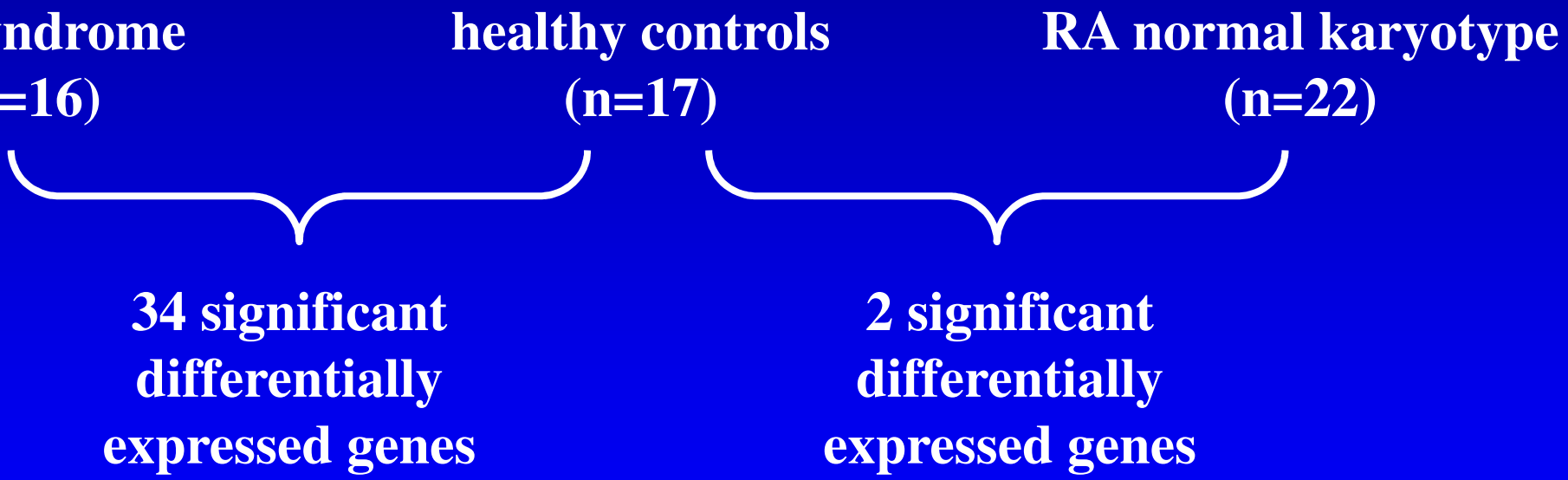
# RPS6 inactivation and the 5q- syndrome

- mice with conditional inactivation of RPS6 display the key features of the 5q- syndrome: macrocytic anaemia, erythroid hypoplasia, megakaryocyte dysplasia with thrombocytosis
- RPS6 hemizygosity caused p53 activation in bone marrow cells
- shows that reduced expression of another ribosomal protein in the mouse results in p53 activation and a 5q- syndrome phenotype
- mice with marked reduction in MDM2 or with pharmacologically induced p53 show erythrocyte and megakaryocyte abnormalities similar to those caused by RPS6 hemizygosity

McGowan et al, Blood, 2011

# Analysis of p53 target genes

- examined expression of 129 p53 target genes (Riley et al, Nat Rev Mol Cell Biol 2008) in bone marrow CD34+ cells using Affymetrix HG133 Plus2.0 arrays




- in RA subtype 5q- syndrome there is significant deregulation of multiple p53 target genes

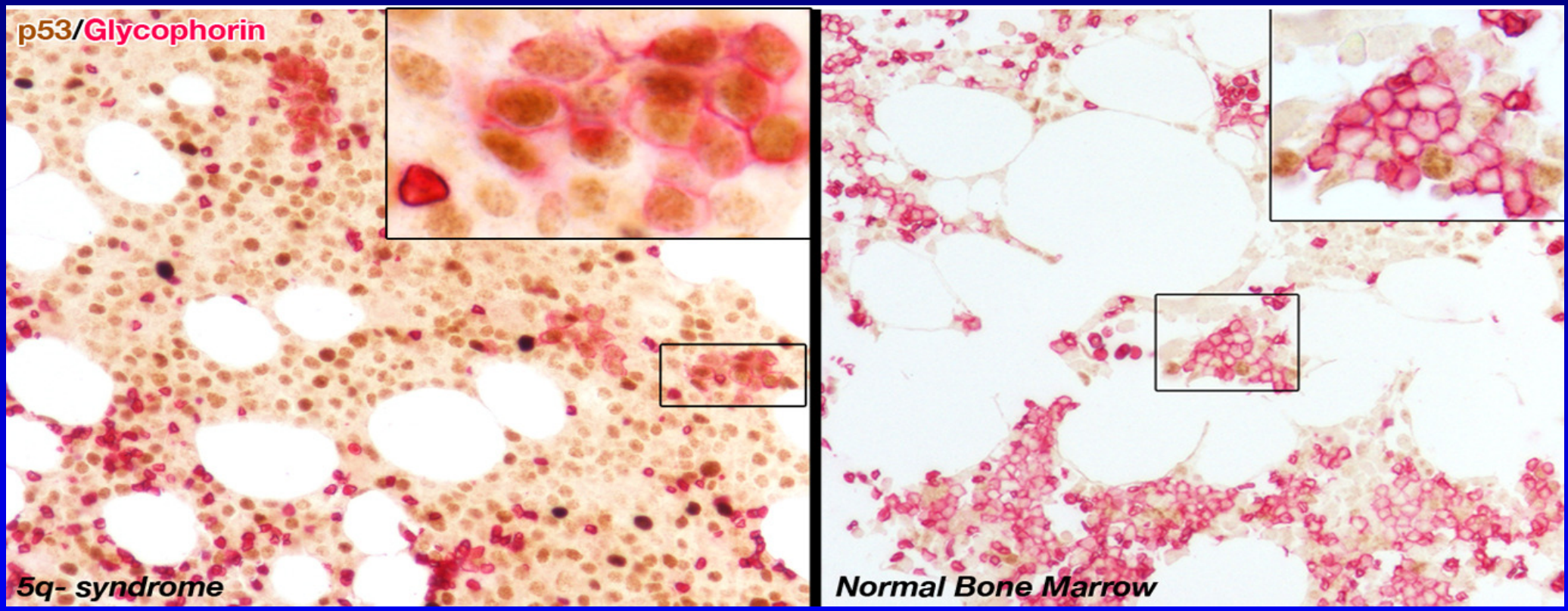
# Analysis of p53 target genes

- 34 significant differentially expressed p53 target genes in 5q- syndrome patients
- determined which cellular processes they were involved in

| | |
|---|---|
| Apoptosis | 15 |
| Cell cycle control | 6 |
| Extracellular matrix | 4 |
| DNA repair | 2 |
| Cytoskeleton | 2 |
| Regulation by p53 upon other signalling pathways | 2 |

- p53 target genes deregulated in 5q- syndrome are mainly involved in apoptosis

# Double immunostaining for p53 and Glycophorin A and C in 5q- syndrome and in normal bone marrow trephines




- in 5q- syndrome (n=7), we observed clusters of p53-positive erythroblasts
- in normal bone marrow, clusters of erythroblasts were p53-negative
- most probably reflects p53 activation secondary to ribosomal stress caused by haploinsufficiency of RPS14

Pellagatti et al, Blood 2010

# RPS14 haploinsufficiency activates p53

- accumulation of p53 protein and increased apoptosis occurs selectively in the erythroid lineage following shRNA knockdown of RPS14 in human HSC in vitro
- GEP analysis (GSEA) showed an up-regulation of multiple p53 target genes eg p21, BAX, WIG1
- pharmacological inhibition of p53 using pifithrin-alpha rescued the erythroid defect in culture

(Dutt *et al*, Blood 2010)

# Ribosomopathies: disorders of ribosome dysregulation

| Disease | Gene defect | Clinical features | Cancer risk |
|---|---|---|---|
| Diamond Blackfan anaemia | RPS19,RPS24,RPS17, RPL35A | Macrocytic anaemia<br>Short stature<br>Craniofacial defects | ?Osteosarcoma<br>?MDS |
| Cartilage Hair Hypoplasia | RMRP | Hypoplastic anaemia<br>Short limbed dwarfism<br>Hypoplastic hair | Non-Hodgkin's lymphoma<br>Basal cell carcinoma |
| Shwachmann-Diamond Syndrome | SBDS | Neutropenia/infections<br>Pancreatic insufficiency<br>Short stature | MDS and AML |
| X-linked Dyskeratosis Congenita | DKC1 | Cytopenias<br>Skin hyperpigmentation<br>Nail dystrophy | AML<br>Head and neck tumours |
| 5q- Syndrome | RPS14 | Macrocytic anaemia<br>Hypolobulated micromegakaryocytes | 10% progression to AML |

# Ribosome dysfunction in the 5q- syndrome

- Introduction and mapping of the CDR
- Role of haploinsufficiency of RPS14 and p53 activation in molecular pathogenesis
- Defective translation and treatment with L-leucine
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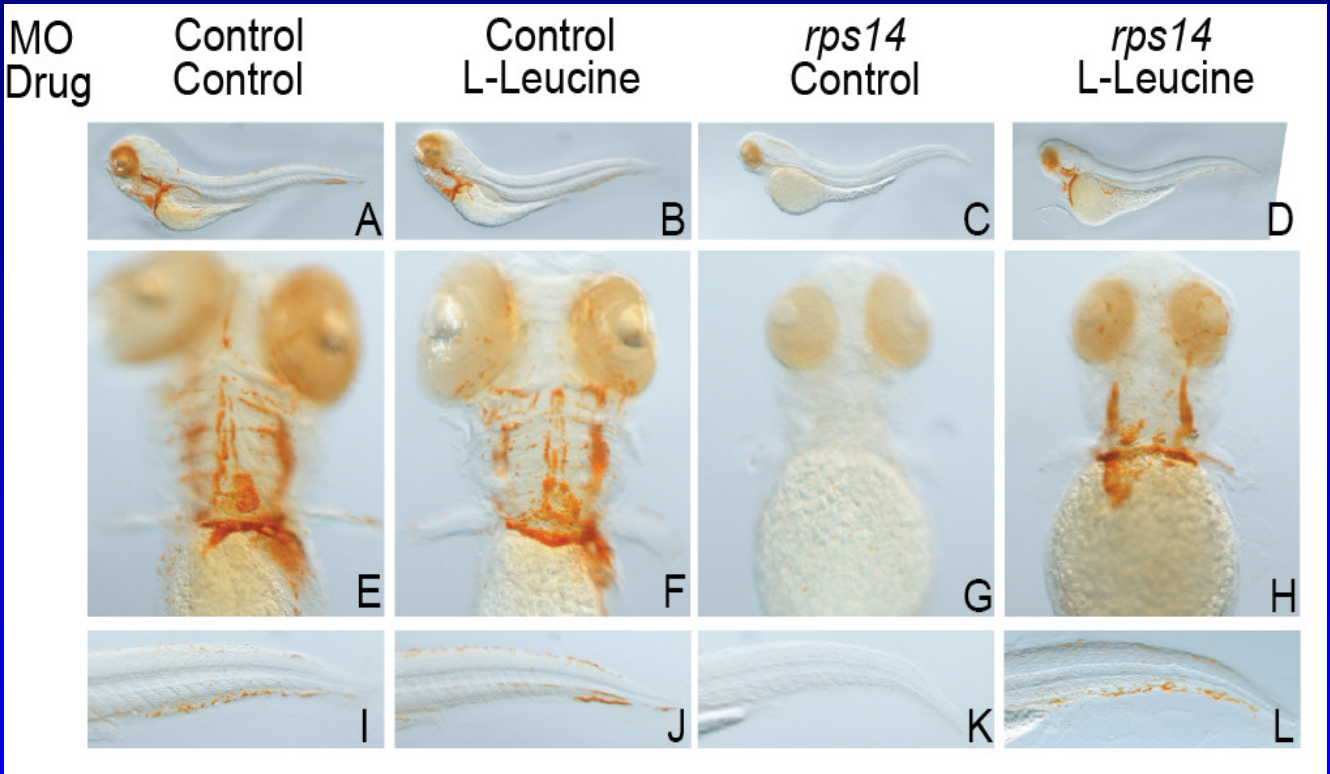
# Translation efficiency

- defective ribosome biogenesis results in a reduction in the efficiency of mRNA translation
- cultured cells from patients with DBA show reduced translational efficiency (Cmjlova et a, Haematologica, 2006)
- this defect in translation may represent a potential therapuetic target in the ribosomopathies

# L-leucine: a translation enhancer

- L-leucine: amino acid that modulates protein synthesis by enhancing translation (activates mTOR pathway and downstream targets, activates translation signaling factors)
- L-leucine shown to improve haemoglobin levels and transfusion independence in patients with DBA (Pospisilova et al, Haematologica, 2007)
- L-leucine supplement partially rescues the erythrocyte and leukocyte numbers in RPS19-deficient mice (Jaako et al, ASH 2011-Abstract 727 Monday, December 12, 2011: 4:30 PM)
- treatment of zebrafish models of DBA and 5q- syndrome with L-leucine resulted in partial reversal of the anaemia (Virgilio et al, ASH 2010, 2011-Abstract 970 Tuesday, December 13, 2011: 8:15 AM)

# L-Leucine improves hemoglobinization and increases the total number of erythrocytes in *rps14* zebrafish morphants




Courtesy of Arati Khanna-Gupta and Beth Payne (ASH 2011, abstract 970)

(A-L) zebrafish embryos at 4dpf stained for hemoglobin with *O*-dianisidine. Lateral views (A-D), ventral (E-H), and tail (I-L).
Control morphants (A, E, I) showed normal hemoglobinization, as did the control morphants treated with leucine (B, F, J). *rps14* antisense morphants - *rps14* haploinsufficient (C, G, K) were severely anemic and exhibited morphological abnormalities. Anemia and developmental defects and were alleviated following leucine treatment (D, H, L)

# Translation efficiency in 5q- syndrome

- haploinsufficiency of RPS14 results in a pre-rRNA processing defect and in 40S subunit deficiency in human cells -it is therefore probable that translation is compromised in these cells
- aim to investigate the effects of L-leucine on erythropoiesis in the 5q- syndrome
- determine whether the use of L-leucine leads to enhanced translation and a rescue of the erythroid defect in cultured cells from 5q- syndrome patients (complementary to similar studies in 5q- mouse model)

# Treatment of 5q- syndrome CD34+ cells with L-leucine

- **experiments are being performed in two stages using:**

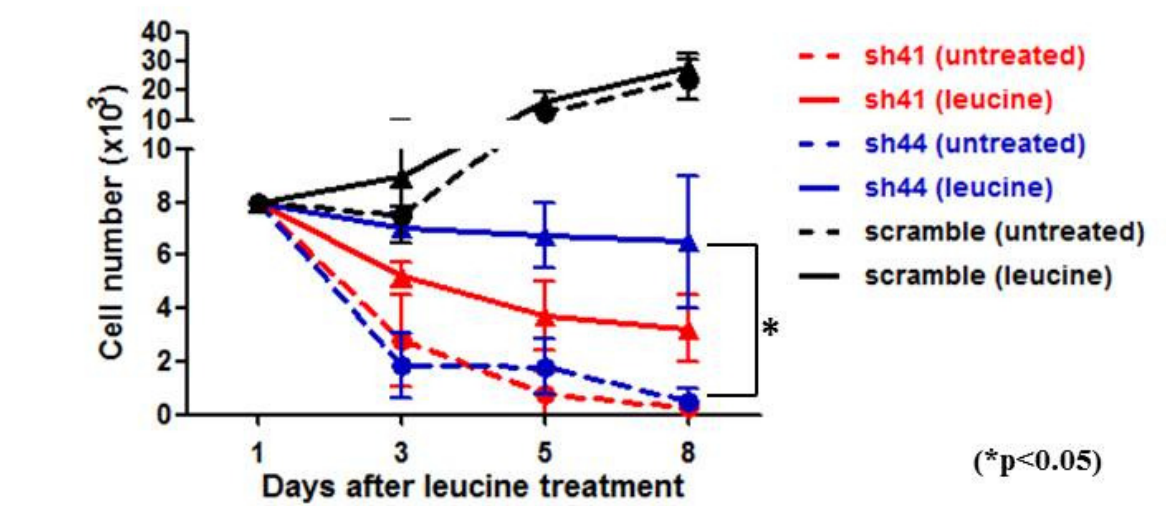
  **cellular model system of 5q- syndrome: lentiviral-based shRNA knockdown of RPS14 has been performed in human CD34+ cells as they differentiate into erythroid cells**

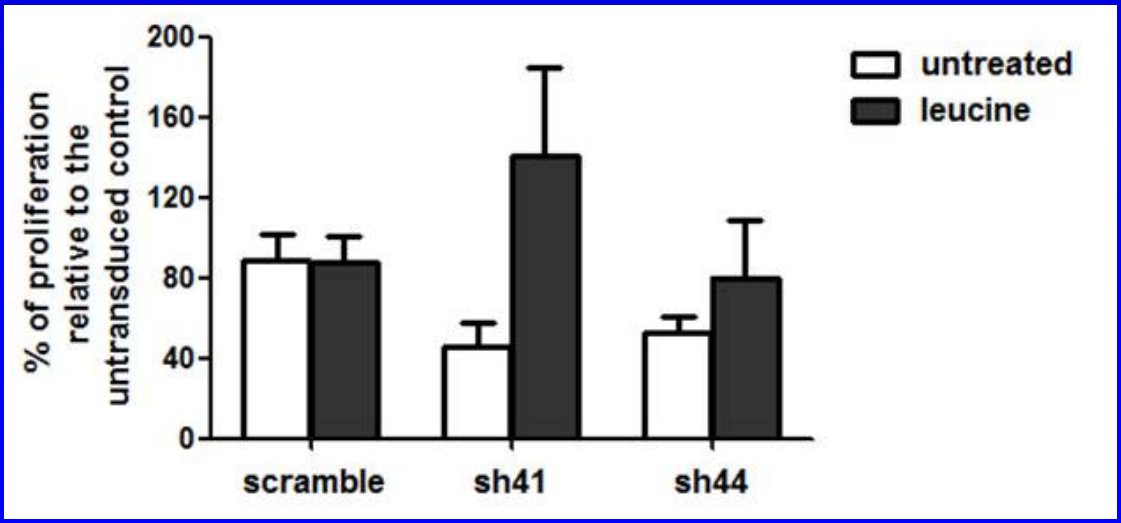
  **5q- syndrome CD34+ cells differentiated into erythroid cells in culture**

# Treatment of RPS14-deficient CD34+ cells with L-leucine

- CD34+ cells from healthy controls were cultured according to the erythroblast culture method (Tehranchi et al, Blood 2005)
- cells were infected with a lentivirus delivering shRNA sequence targeting the RPS14 gene, resulted in ~50% knockdown
- L-leucine (600 µg/ml) was added at day 7
- determining the effects of L-leucine treatment on erythroid differentiation, cell growth, apoptosis, translation and mTOR activation at day 11

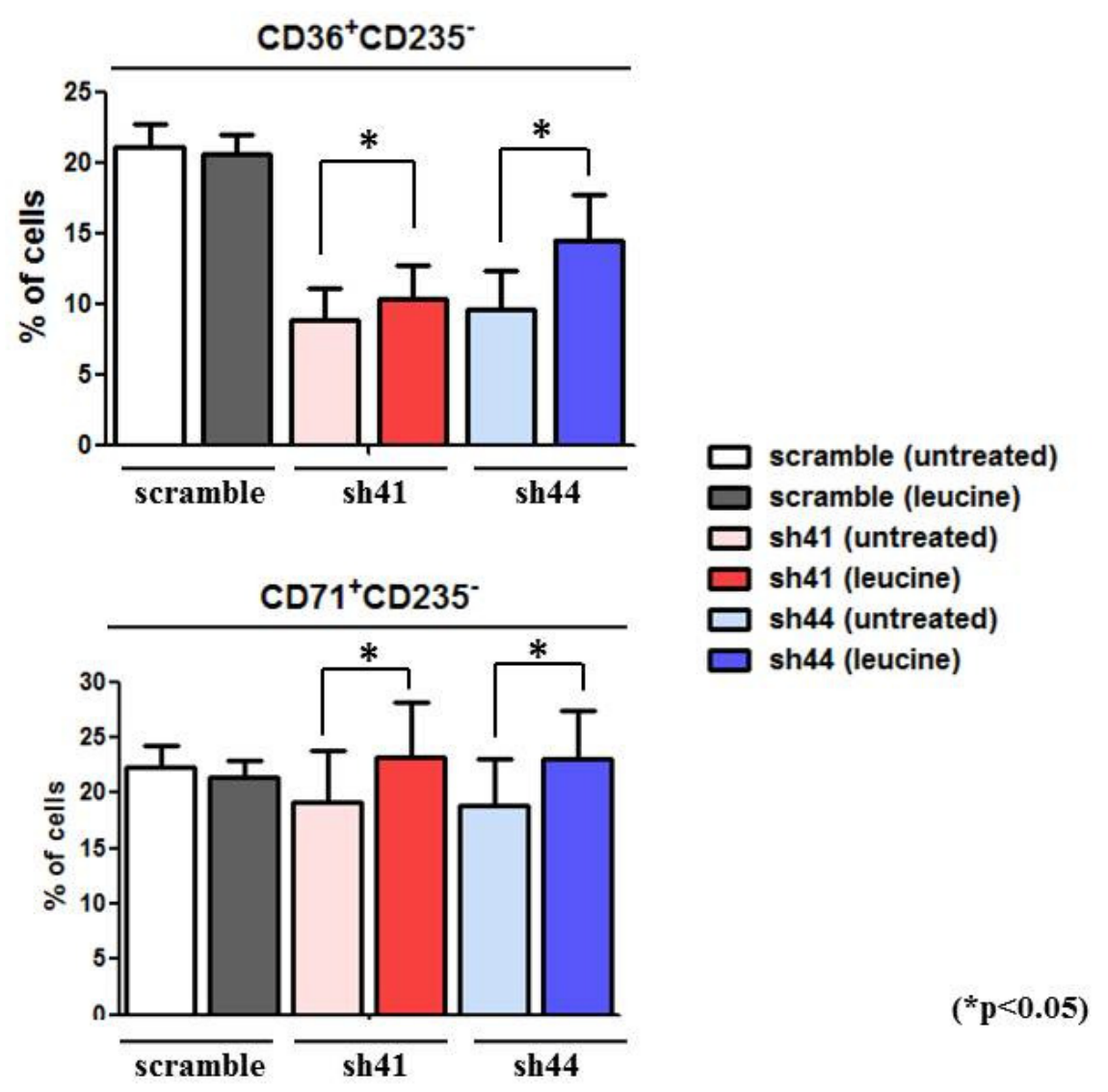
# Treatment with leucine improves cell expansion due to increase in proliferation



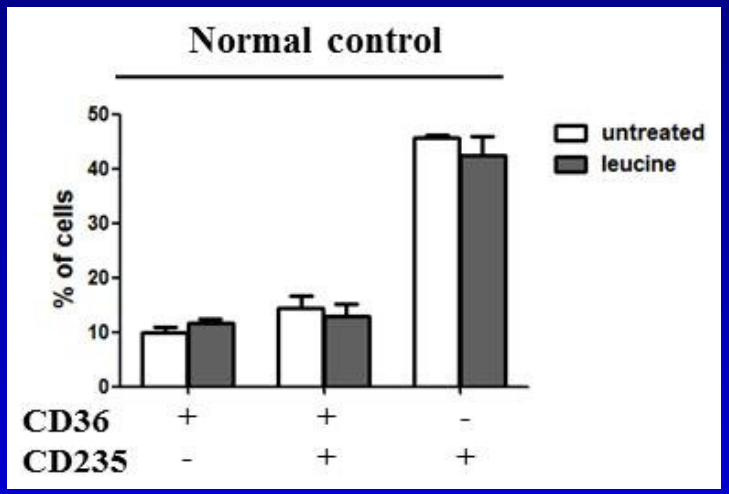

- Treatment with L-leucine results in a significant increase in cell number in cells with RPS14 knockdown
- Treatment with L-leucine does not increase cell number in the scramble control
- Treatment with L-leucine increases proliferation (MTS assay) in cells with RPS14 knockdown
- No difference is observed in the scramble control

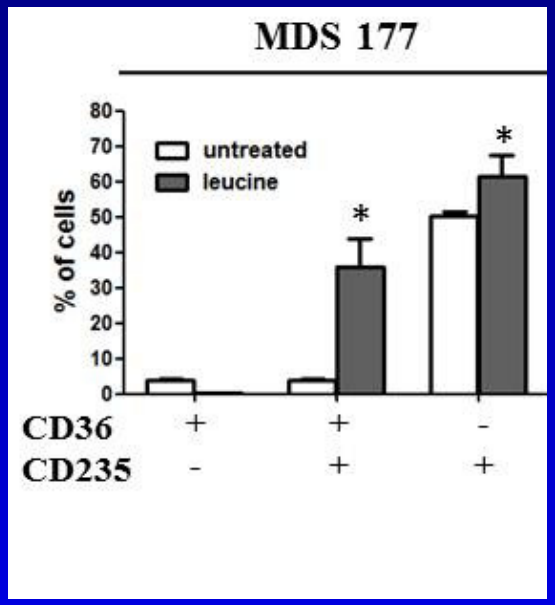
# Stimulation of erythroid differentiation after L-leucine treatment -RPS14-deficient cells

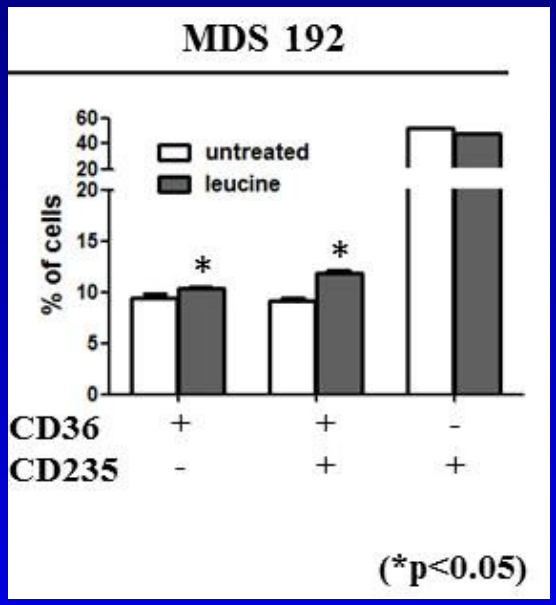



- There is a significant increase in the % of CD36+ CD235- cells and CD71+ CD235- cells at day 11 in cells with RPS14 knockdown following treatment with L-leucine
- Treatment with L-leucine does not increase erythroid differentiation in the scramble control

# Stimulation of erythroid differentiation after leucine treatment- 5q- syndrome cells



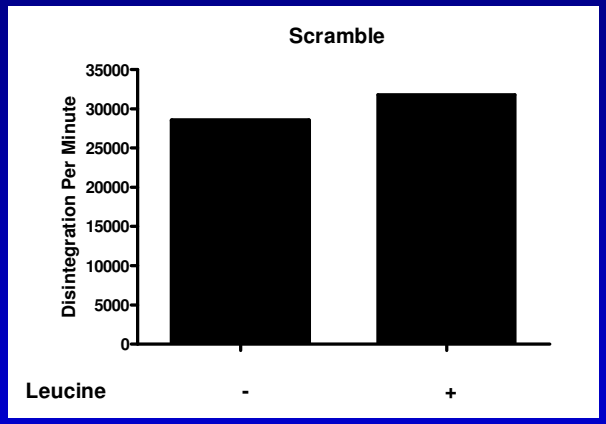

- Treatment with L-leucine results in a significant increase in the % of differentiating erythroid cells (as assessed by CD36 CD235 staining) in cultured cells from patients with 5q- syndrome at day 11

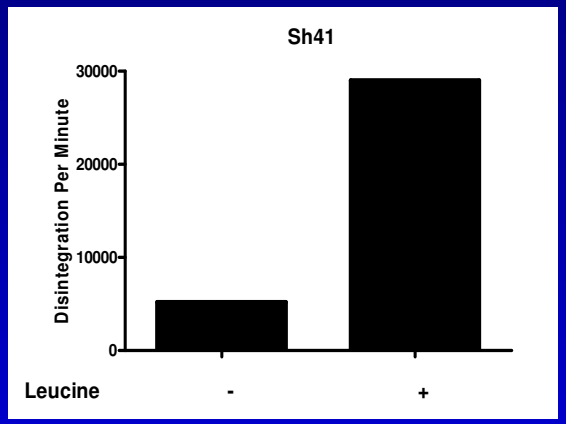
- Treatment with L-leucine has no significant effect on erythroid differentiation in cultured cells from healthy controls

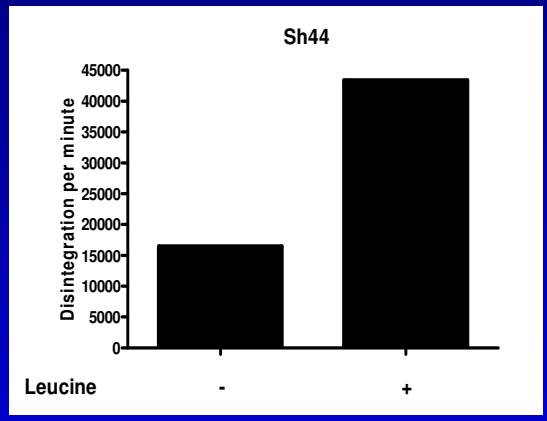
# L-leucine improves translation efficiency

- Translation efficiency was measured by incorporation of L-[3H]-leucine into proteins

RPS14 knockdown cells

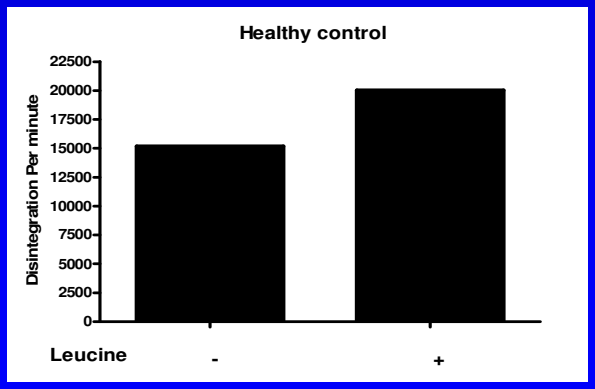


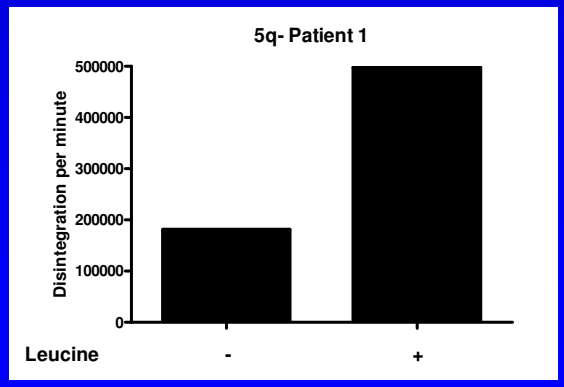



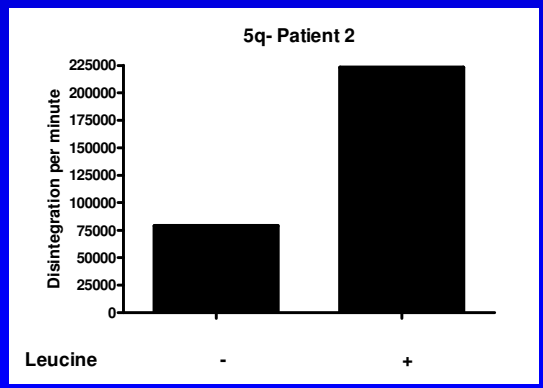




- in the cells with RPS14 knockdown L-leucine increases translation levels by 2.6-5.6 fold (scramble control: 1.1 fold)

5q- syndrome patient cells




- in the two 5q- patient cells L-leucine increases translation levels by 2.7-2.8 fold (healthy control cells: 1.3 fold)

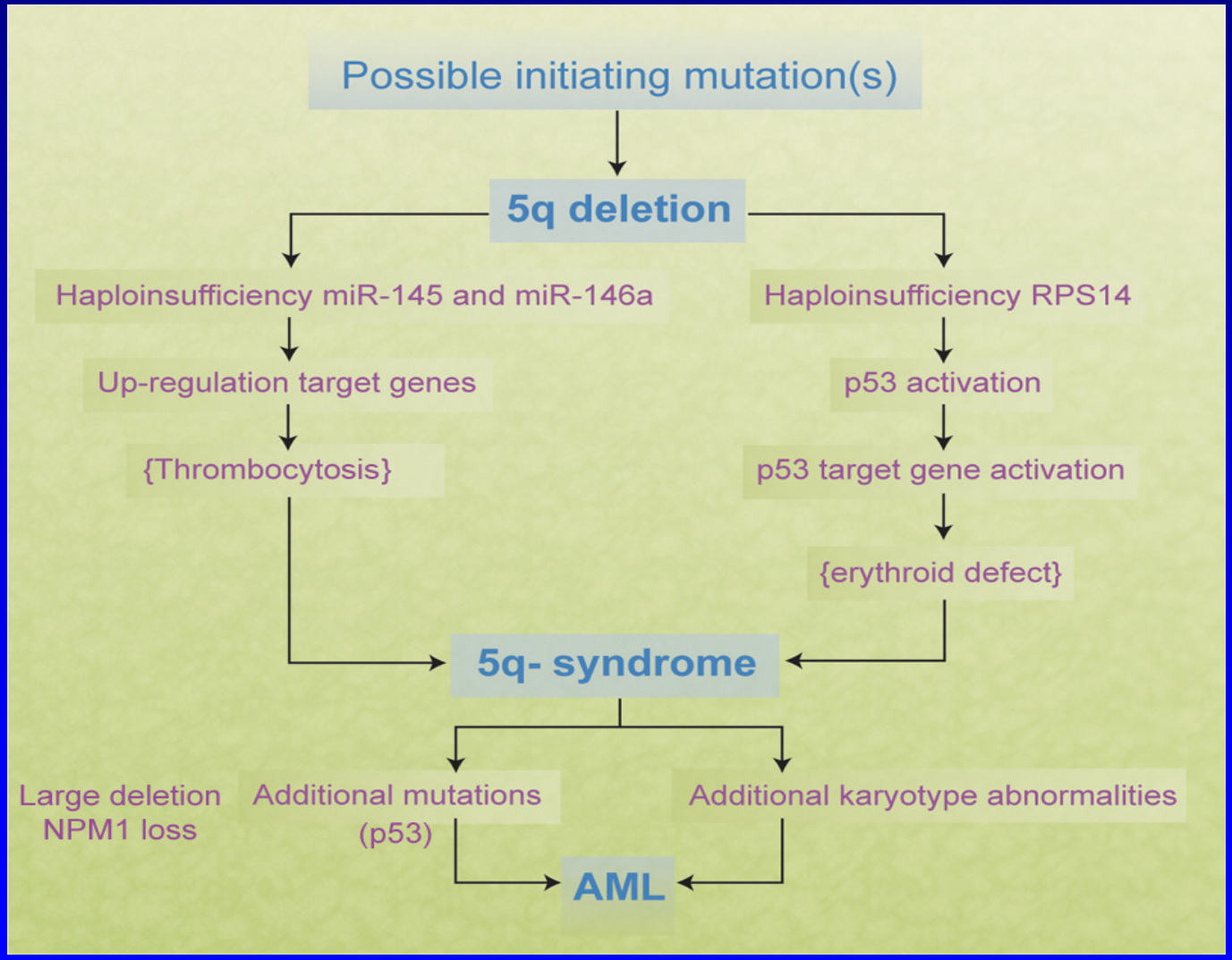
# L-leucine in the treatment of RPS14-deficient cells

- Studies of a zebrafish model of the 5q- syndrome, RPS14-deficient human erythroid cells, and cultured erythroid cells from patients with the 5q- syndrome support the consideration of leucine as a therapy for the 5q- syndrome

# Ribosome dysfunction in the 5q- syndrome

- Introduction and mapping of the CDR
- Role of haploinsufficiency of RPS14 and p53 activation in molecular pathogenesis
- Defective translation and treatment with L-leucine
- Cooperating genetic events in disease pathogenesis

# Cooperating genetic events in the development of the 5q- syndrome and progression to AML

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