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ORIGINAL ARTICLE

A flow cytometry-based reporter assay identifies macrolide antibiotics as nonsense mutation read-through agents

Michal Caspi[1] · Anastasia Firsow[1] · Raja Rajkumar[1] · Nir Skalka[1] · Itay Moshkovitz[1] · Ariel Munitz[1] · Metsada Pasmanik-Chor[2] · Hagar Greif[3] · Dalia Megido[3] · Revital Kariv[4] · Daniel W. Rosenberg[5] · Rina Rosin-Arbesfeld[1]

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Abstract
A large number of human diseases are caused by nonsense mutations. These mutations result in premature protein termination and the expression of truncated, usually nonfunctional products. A promising therapeutic strategy for patients suffering from premature termination codon (PTC)-mediated disorders is to suppress the nonsense mutation and restore the expression of the affected protein. Such a suppression approach using specific antibiotics and other read-through promoting agents has been shown to suppress PTCs and restore the production of several important proteins. Here, we report the establishment of a novel, rapid, and very efficient method for screening stop-codon read-through agents. We also show that, in both mammalian cells and in a transgenic mouse model, distinct members of the macrolide antibiotic family can induce read-through of disease-causing stop codons leading to re-expression of several key proteins and to reduced disease phenotypes. Taken together, our results may help in the identification and characterization of well-needed customized pharmaceutical PTC suppression agents.

Key messages
- Establishment of a flow cytometry-based reporter assay to identify nonsense mutation read-through agents.
- Macrolide antibiotics can induce read-through of disease-causing stop codons.
- Macrolide-induced protein restoration can alleviate disease-like phenotypes.

Keywords Nonsense mutations · Premature termination codons (PTCs) · Genetic diseases · Ribosomal read-through · Aminoglycoside and macrolide antibiotics

Introduction

Premature termination codons (PTCs) impede protein production by causing the ribosome to release the premature peptide, which is usually nonfunctional and thus marked for degradation. In addition, the messenger RNA containing a nonsense mutation is often rapidly degraded through the process of nonsense mutation-mediated decay [1–3]. Nonsense mutations lead to a large range of human diseases [4, 5]. The first evidence regarding the ability of certain antibiotics to alter ribosome activity in a way that overcomes premature termination was presented three decades ago by Burke and Mogg [6]. Genetic and biochemical studies have since then shown that aminoglycoside antibiotics bind a specific site in ribosomal RNA and as a result, the ribosome introduces a missense mutation and translates through the termination codon. Different synthetic molecules also have the ability to

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✉ Rina Rosin-Arbesfeld
arina@post.tau.ac.il

[1] Department of Clinical Microbiology and Immunology, Sackler School of Medicine, Tel Aviv University, Ramat-Aviv, 69978 Tel Aviv, Israel

[2] Bioinformatics Unit, G.S.W. Faculty of Life Science, Tel Aviv University, Tel Aviv, Israel

[3] Bioblast Pharma, Tel Aviv, Israel

[4] Department of Gastroenterology, Tel Aviv Sourasky Medical Center, Tel Aviv 64239, Israel

[5] Center for Molecular Medicine, University of Connecticut School of Medicine, Farmington, CT, USA

read-through stop codons [7]. It is important to note that read-through of PTCs is much more efficient than read-through of wild-type stop codon, as the latter is positioned near the poly(A) tail that promotes ribosomal release while the random positioning of the nonsense mutations frequently places them at a significant distance from the poly(A) tail. Moreover, there is evidence for prolonged ribosomal pausing at nonsense mutation sites which may make them susceptible to drug-induced suppression [8]. Additionally, it has been reported that read-through levels depend on different parameters, not all known, such as the type of the stop codon and the stop codon surrounding nucleotide context [9–12]. We have previously shown that members of macrolide antibiotics can induce PTC read-through of the adenomatous polyposis coli (APC) gene [13]. Over all, nonsense mutations are responsible for anywhere between 5 and 70 % of individual cases of most inherited diseases. Among them are cystic fibrosis (CF), Duchene muscular dystrophy (DMD), Usher syndrome (USH), RETT syndrome (RTT), and numerous types of cancer. In all these cases, the disease is a result of premature termination of key proteins. Thus, induced production of a full-length protein, even if only in a limited capacity, may be functionally and therapeutically significant. This is especially relevant for disorders where protein expression is essentially absent, such as CF, spinal muscular atrophy (SMA), ataxia-telangiectasia (A-T), and others. In some cases, such as in lysosomal storage disease, even 1 % of normal protein function may restore a near-normal or clinically less severe phenotype [14, 15]. However, this threshold is disease dependent; as for CF, it has been shown that 10 to 35 % of CFTR activity might be needed to significantly alleviate pulmonary morbidity [16]. A large number of different antibiotic and non-antibiotic compounds are being tested for their ability to induce stop-codon read-through [17, 18]. Thus, we have developed a novel vector that allows rapid and efficient screening of distinct drug compounds for their ability to induce PTC suppression. Since a recent study revealed that the luciferase-based assays traditionally used for screening read-through agents may have off-target effects [19], we utilized two fluoro-proteins and flow cytometry analysis. This method allows quick and effective large-scale screening of different compounds. Our results, using this reporter plasmid, show that, as previously published, members of the aminoglycoside and macrolide antibiotic families can induce read-through of nonsense mutations. We also show that these antibiotic compounds can lead to restoration of numerous key proteins that are involved in different diseases and in cancer. Experiments in mice show that antibiotic treatment can reduce intestinal polyp burdens that result from PTC mutations in the APC gene, leading to large changes in overall gene expression.

Materials and methods

Cell cultures Human embryonic kidney (HEK293T) and human colorectal adenocarcinoma (SW480 and SW1417) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 100 U/ml penicillin-streptomycin. Cells were kept in a humidified 5 % CO2 atmosphere at 37 °C. Cells were transfected using JetPEI with 1 μg of reporter plasmid that contains the sequence of a nonsense mutation. Twenty-four hours later, the medium was replaced with medium-containing read-through agents and the incubation was repeated for 24 h. For "proof-of-concept" assays, β-lymphocytes derived from A-T patients carrying a mutation in codon 5515 (The Coriell Cell Repositories#GM11264); WT β-lymphocytes, a gift from Dr. Yossi Shiloh; RETT syndrome fibroblasts containing the nonsense mutation 880C>T (R294X), a gift from Dr. Bruria Ben-Zeev; SMA patients' fibroblasts from The Coriell Cell Repositories #GM09677 (SMN1−/−; SMN2+/−) and #GM00232 (SMN1−/−; SMN2+/+); WT fibroblast from The Coriell Cell Repositories#GM08680. All cells were grown according to supplier's instructions. As tested in numerous experiments, streptomycin had no effect on read-through levels.

Antibodies and reagents Anti-ATM (rabbit monoclonal—D2E2; Cell Signaling #2873; WB-1:1000), anti-MeCP2 (mouse monoclonal—Mec-168; abcam #ab50005; WB and IF-1:1000), anti-SMN (mouse monoclonal; BD #610646; WB-1:5000), anti-APC (mouse monoclonal; Abcam, 1:300), anti-active β-catenin (mouse monoclonal; Millipore, WB 1:1000 and IF 1:300), anti-phospho-β-catenin (rabbit polyclonal; Cell Signaling, WB 1:1000 and IF 1:300), anti-Tubulin (mouse monoclonal; Sigma, 1:10000), anti-GFP (rabbit; Santa Cruz; WB-1:1000), anti-mouse and anti-rabbit-HRP (Jackson Laboratories, 1:10,000), Alexa-488 and 596 secondary (Molecular Probes, 1:500), DRAQ5 (Cell Signaling, 5 μM), DAPI (Sigma 10 μg/ml), azithromycin (Pfizer SH0609), erythromycin (Sigma E5389), and gentamicin sulfate (Biological Industries 03-035). PTC124 was obtained from AdooQ BioScience, LLC (catalog no. A10758-50).

Plasmid design pEGFP-C2-BFP was constructed by inserting the BFP ORF into the pEGFP-C2 vector (catalog number 6083-1; Invitrogen) using the KpnI and HindIII restriction enzymes (for the full map, see supplementary data). Complimentary oligonucleotides that contain wild-type or mutated sequence of different mutations were inserted in

frame to the pEGFP-C2-BFP vector between GFP and BFP reporter genes using XhoI and EcoRI HF restriction enzymes. The upstream GFP gene serves as control of transfection efficiency and the downstream BFP gene indicates the PTC read-through efficiency.

C > T
CAAACAGCTCAAACCAAG**T**GAGAAGTACCTAAAA-ATAAAGCA APC (R1450X)

G > T
CTGCAAATAGCAGAAATAAAA**T**AAAAGATTGGAAC-TAGGTCA APC (E1309X)

T > G
CTTCCTCTCCTCATCCAGCTTT**G**ACATGGCAATGAC-AAAGAC APC (L360X)

T > A
TCTGAGAAAGACAGAAGTT**A**GGAGAGAGAGCGAG-GTATTGGC mAPC (L850X)

C > T
AAATTTAAGCGCCTGATTTGAGATCCTGAAACAATT-AAACAT ATM (R35X)

C > T
GTGAAAACTGACTTTTGT**T**AGACTGTACTTCCATAC-TTGATT ATM (Q1839X)

C > T
CTCTATGATGTGCTG**T**GAATGTACCACCAGACCATG-GACAAG Ush(R31X)

C > T
AGAGGGAGCCCCTCCCGG**T**GAGAGCAGAAACCAC-CTAAG RTT(R168X)

C > T
GTGGTCTACCCTTGGACC**T**AGAGGTTCTTTGAGTC-CTTTGGG TALA (Q61X)

Flow cytometry The cells treated with read-through agents were trypsinised and washed with PBS, suspend in 300 μl of PBS, transferred to FACS tubes, and analyzed. Events were acquired by a Gallios flow cytometer system (Beckman Coulter, Brea, CA, USA), and data analyzed using the Kaluza (Beckman Coulter) software on at least 5000–10,000 cells.

Immunofluorescence (IF) SW480 cells or fibroblasts were grown on 13-mm round coverslips and treated with antibiotics for 7 days as described. The cells were then fixed for 20 min in PBS containing 4 % paraformaldehyde. After three washes with PBS, the fixed cells were permeabilized with 0.1 % Triton X-100 for 10 min and blocked with bovine serum albumin for 1 h. Subsequently, cells were incubated at room temperature with primary and secondary antibodies for 60 and 30 min, respectively. 4–6′ Diamidino-2 phenylindole (DAPI, Sigma 10 μg/ml) or DRAQ5 were used to stain cell nuclei.

Western blot analysis Cells after antibiotic treatment were washed with PBS and solubilized in lysis buffer (100 mM NaCl, 50 mM Tris, pH 7.5, 1 % Triton X-100, 2 mM EDTA) containing protease inhibitor cocktail (Sigma). Extracts were clarified by centrifugation at 12,000×*g* for 15 min at 4 °C. Following SDS polyacrylamide gel electrophoresis (SDS-PAGE) separation, proteins were transferred to nitrocellulose membranes and blocked with 5 % low fat milk. Membranes were incubated with specific primary antibodies, washed with PBS containing 0.001 % Tween-20 (PBST), and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. After washing in PBST, membranes were subjected to enhanced chemiluminescence detection analysis. Band intensity was measured by TINA analysis software.

Animal studies Five-week-old C57BL/6JApc$^{Min/+}$ mice were treated with intraperitoneal injection (IP) of 600 μg erythromycin 5 days a week for 4 months. At the end of the treatment, the mice were sacrificed and intestines were removed for RNA analysis using Illumina Direct Hyb Assay or polyps counting using 0.5 % methylene blue staining. Genomic DNA was prepared from tail-snips, using Tail Lysis Buffer (10 mmol/l Tris pH 8, 100 mmol/l NaCl, 25 mmol/l EDTA, 0.5 % SDS) and 10 mg/ml Proteinase K (Roche). Offspring were characterized for the Min genotype by PCR using specific primers as previously described [20].

Illumina array

RNA was extracted from intestinal mucosa of 10 control and 10 treated Apc$^{Min/+}$ mice using TRI Reagent (SIGMA-ALDRICH) and subjected to Illumina Direct Hyb Assay. The results were analyzed using the KEGG pathway analysis (using David functional enrichment tool) [21]

Results

A GFP-BFP reporter plasmid

Previous works from our laboratory and others have utilized a dual luciferase reporter plasmid system to measure the ability of different compounds to induce stop-codon read-through [13, 22–28]. However, a recent study has shown that this method may be somewhat biased towards different compounds [19]. Thus, we have developed a technique in which the green fluorescent protein (GFP) open reading frame and the blue fluorescent protein (BFP) open reading frame are located on either side of wild-type or stop-codon containing sequences (Fig. 1a). Specific stop codons and the surrounding sequence of known disease-causing nonsense mutations were

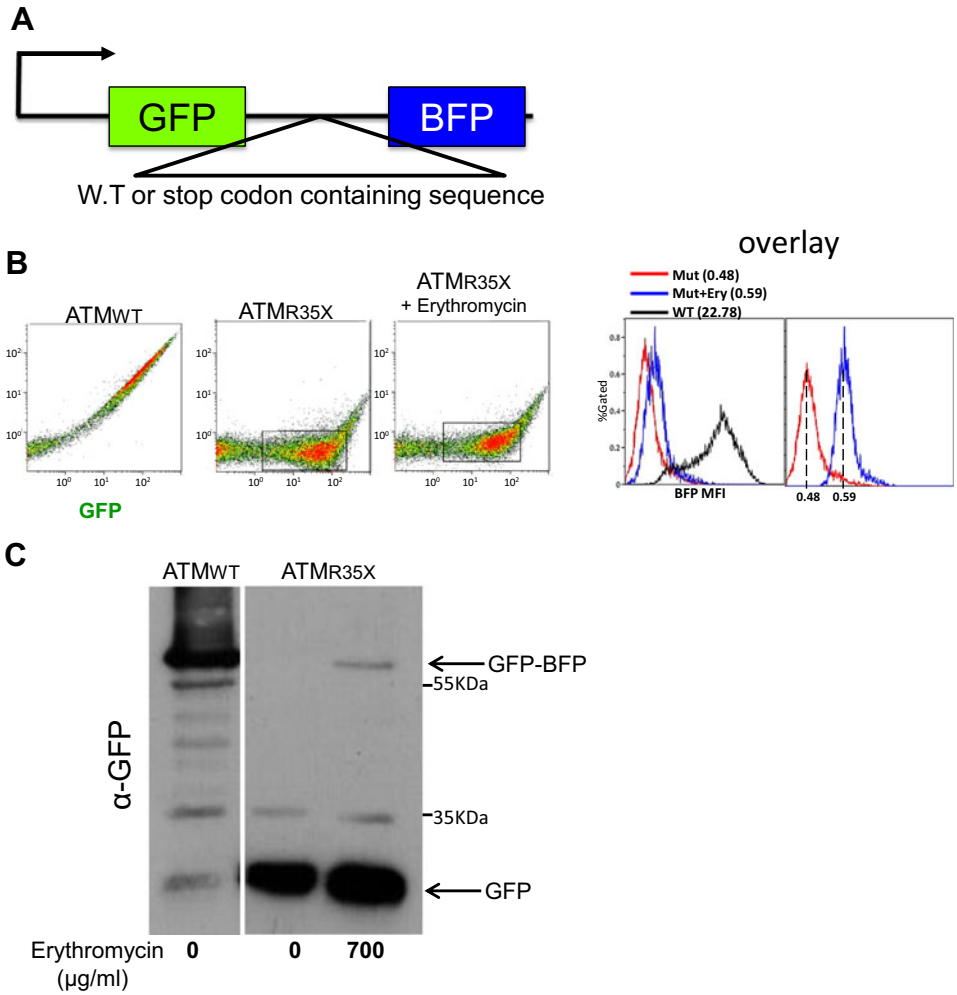
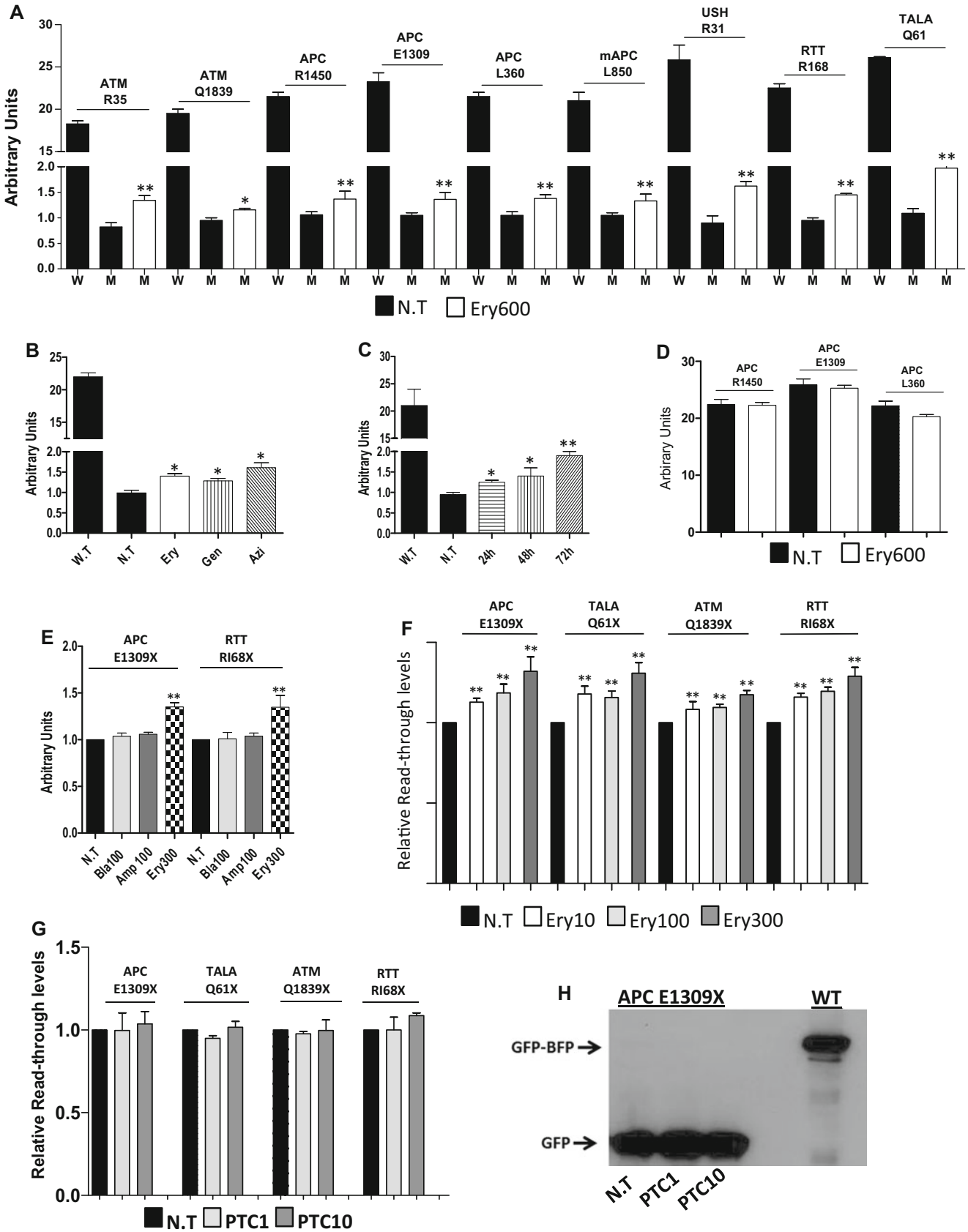


Fig. 1 Plasmid construction and feasibility test. **a** Schematic description of the GFP-BFP construct; WT or mutated (PTC) sequences were introduced into the GFP-BFP plasmid (full map in supplementary data). **b** Representative flow cytometry results; HEK293T cells were transfected with the indicated constructs and subjected to antibiotic treatment as described. Events were acquired by a Gallios flow cytometer system (Beckman Coulter, Brea, CA, USA). BFP-MFI of equally gated double-stained cells is depicted in an overlay plot; Plot is presented with and without WT values. MFI median was transferred to bar graphs in all future experiments. **c** HEK293T cells were transfected with the indicated constructs and subjected to antibiotic treatment as described. Cells were then harvested and analyzed by Western blotting using an anti-GFP antibody

inserted between the GFP and BFP open reading frames. The upstream GFP gene serves as a marker for transfected cells and thus only GFP-labeled cells were collected for analysis. Translation of the BFP originates from the same translation initiation signal but is downstream to the inserted, specific sequence. The levels of BFP and GFP were determined using flow cytometry (FACS). Figure 1b shows the distribution of double-stained cell populations harboring a wild-type or ataxia-telangiectasia-mutated (ATM) 103C>T sequences. The ATM 103 mutant was treated with 700 μg/ml erythromycin for 48 h. Equally gated area of treated and untreated cells was measured and the values are shown in an overlay plot. Treated cells show increased BFP-mean fluorescence intensity (MFI) compared to mutant untreated cells illustrating read-through levels. Note that WT MFI is substantially higher than the mutated sequences (22.78 compared to 0.48 or 0.59) as antibiotic-mediated read-through is known to account for only 10–15 % of protein restoration. All further reporter-based experiments are presented as bar graphs of BFP-MFI median values. Western blot analysis was used to show the expression of the restored reporter protein product (Fig. 1c).

Antibiotic-induced stop codon read-through

We next tested the ability of the GFP-BFP construct to measure PTC read-through of numerus disease-causing mutations by several read-through agents. The different constructs were transfected into HEK293T cells and treated with 600 μg/ml erythromycin for 48 h. Results show that this treatment led to read-through levels of approximately 10 % as compared to the wild-type sequence (Fig. 2a). Several lines of evidence suggest that the efficiency of the stop-codon suppression depends on the stop-codon type (with the ranking order generally being UGA>UAG>UAA) [12] and is influenced by the sequence surrounding the stop codon, especially the +4 nucleotide [10, 11]. Recently, it has also been shown that a uracil residue immediately upstream to the stop codon is a major determinant of the response to gentamicin [12]. Our results, using nine different sequences (Table 1) show that the read-through agent's treatment led to stop-codon suppression of all sequences tested with varied efficiencies, but with no apparent consensus. Additional experiments revealed that erythromycin concentrations ranging from 100 to 600 μg/ml


A
Arbitrary Units
ATM R35
ATM Q1839
APC R1450
APC E1309
APC L360
mAPC L850
USH R31
RTT R168
TALA Q61
W M M
N.T Ery600
B
Arbitrary Units
W.T N.T Ery Gen Azi
C
Arbitrary Units
W.T N.T 24h 48h 72h
D
Arbirary Units
APC R1450
APC E1309
APC L360
N.T Ery600
E
Arbitrary Units
APC E1309X
RTT RI68X
N.T Bla100 Amp 100 Ery300 N.T Bla100 Amp100 Ery300
F
Relative Read-through levels
APC E1309X
TALA Q61X
ATM Q1839X
RTT RI68X
N.T Ery10 Ery100 Ery300
G
Relative Read-through levels
APC E1309X
TALA Q61X
ATM Q1839X
RTT RI68X
N.T PTC1 PTC10
H
APC E1309X
WT
GFP-BFP
GFP
N.T PTC1 PTC10

◀ **Fig. 2** Testing the effect of distinct parameters on read-through activity. **a** Erythromycin treatment of nine nonsense mutations. HEK293T cells were transfected with the indicated constructs mutated (*M*) or wild-type (*WT*) sequences and were untreated (*NT*) or treated with 600 μg/ml erythromycin for 48 h as indicated. The cells were then collected and analyzed by flow cytometry. **b** Comparison of different antibiotics. HEK293T cells were transfected with the APC (R1450X) construct and treated with gentamycin (*Gen*), erythromycin (*Ery*), and azithromycin (*Azi*) 100 μg/ml for 48 h. The cells were collected and analyzed by flow cytometry. **c** Erythromycin treatment with different incubation periods. HEK293T cells were transfected with the APC (R1450X) construct and treated with erythromycin (100 μg/ml) for 24, 48, and 72 h. **d** WT sequences are not affected by read-through agents. WT sequences of the three APC mutants were transfected as described and treated with 600 μg/ml erythromycin for 48 h. **e** Antibiotics that do not affect the ribosome have no read-through activity. HEK293T cells were transfected with the APC (E1309X) and RTT (R168X) sequences and treated with the indicated antibiotics. Analysis was performed as before. *Bla* blasticidin, *Amp* ampicillin. **f** Dose-dependent assay. HEK293T cells were transfected with the indicated constructs and treated with increasing concentrations of erythromycin (10, 100, 300 μg/ml). **g** PTC124 dose-dependent assay. HEK293T cells were transfected with the indicated constructs and treated with increasing concentrations of PTC124 (1, 10 μg/ml). Growth media was depleted of penicillin-streptomycin during treatment. **h** WB analysis of HEK293T cells transfected with the APC construct (mutated or WT sequences) and treated with PTC124 as indicated. In all cases, the BFP-MFI median values are shown as mean± SD from at least five independent experiments performed in duplicates. *Asterisks* denote statistical significance of treated samples as compared to untreated samples in an unpaired Student's *t* test. **P*≤0.05; ***P*≤0.01; ***P*≤0.008

resulted in similar read-through levels (not shown). We then used 100 μg/ml of erythromycin, gentamicin, and azithromycin for 48 h on cells transfected with the APC (R1450X) construct. Our results demonstrate that all tested antibiotics induced approximately similar read-through levels (Fig. 2b). A time-dependent experiment using 100 μg/ml erythromycin revealed that 72 h treatment led to higher read-through levels as compared to 24- and 48 h treatments (Fig. 2c). The WT sequences of the three APC mutations were tested to assure treatment specificity. Indeed, erythromycin (600 μg/ml) had no effect on read-through measurements (Fig. 2d). We also verified that other antibiotics (that do not affect the ribosome) have no read-through capacity (Fig. 2e). Finally, we have conducted a dose-dependent assay using erythromycin (Fig. 2f). The data depicts a minor dose-dependent read-through increase for the different erythromycin concentration. A number of reports, using different systems, claimed that PTC124 does not induce stop-codon read-through [19, 29–35]. Nonetheless, other studies have shown that PTC124 does harbor read-through capabilities [36–40], indicating that the results may reflect differences between mutations types, compounds, and working techniques. Our results, using the GFP-BFP flow cytometry method, show that under the tested concentrations, PTC124 had no effect on stop-codon read-through levels (Fig. 2g). The raw data of this experiment is shown as SI Fig. 2. Although a tenfold higher concentration of PTC124 had some affected on the BFP levels, no restoration of the GFP-BFP fusion protein could be detected by Western blot analysis (not shown).

Table 1 Nine different sequences show that the read-through agent's treatment led to stop-codon suppression of all sequences tested with varied efficiencies, but with no apparent consensus

Mutation	−1 nucleotide	Stop codon	+4 nucleotide
APC R1450X	G	UGA	G
APC E1309X	U	UAA	A
APC L360X	A	UGA	C
Ush R31X	G	UGA	A
ATM R35X	U	UGA	G
RTT R168X	G	UGA	G
TALA Q61X	C	UAG	A
mAPC L850X	T	UAG	G
ATM Q1839X	T	UAG	A

Antibiotic-induced read-through in patient-derived cells

The next step was to corroborate our reporter plasmid data by testing read-through activity within the context of endogenous mutated proteins derived from afflicted patients. Here, we present data regarding three orphan diseases: ataxia-telangiectasia (A-T), RETT syndrome (RTT), and spinal muscular atrophy (SMA). A-T is an autosomal recessive syndrome [41] that is caused by biallelic mutations in ATM, a gene encoding a large protein kinase involved in DNA damage repair [42]. Approximately 14 % of the genetic mutations in patients with A-T are single-nucleotide changes that result in PTCs. Thus, we tested PTC suppression of mutated ATM protein using erythromycin or gentamycin. To accomplish this, we used β-lymphocytes derived from A-T patients carrying the ATM (Q1839X) mutation (Coriell #GM11264). The cells were incubated in medium-containing antibiotics (as indicated) for 7 days. The cells were then harvested and subjected to SDS-PAGE analysis using specific anti-ATM antibodies. Figure 3a (upper panel) shows the restoration of the ATM protein after antibiotic treatment. Band intensity was determined using TINA analysis of two independent experiments (lower panel) .

RETT syndrome can be caused by at least 200 different mutations of the effected gene methyl-CpG binding protein-2 (MeCP2), including missense, nonsense, frame-shifts, and deletions. Four of the five most frequent mutations associated with this disease are nonsense mutations that are found in almost a fourth of all patients [43]. To test the efficiency of antibiotic-mediated read-through of the MeCP2 protein, we used fibroblasts derived from a RETT patient carrying the nonsense mutation, R294X. The cells were treated as described and subjected to Western blotting using a specific

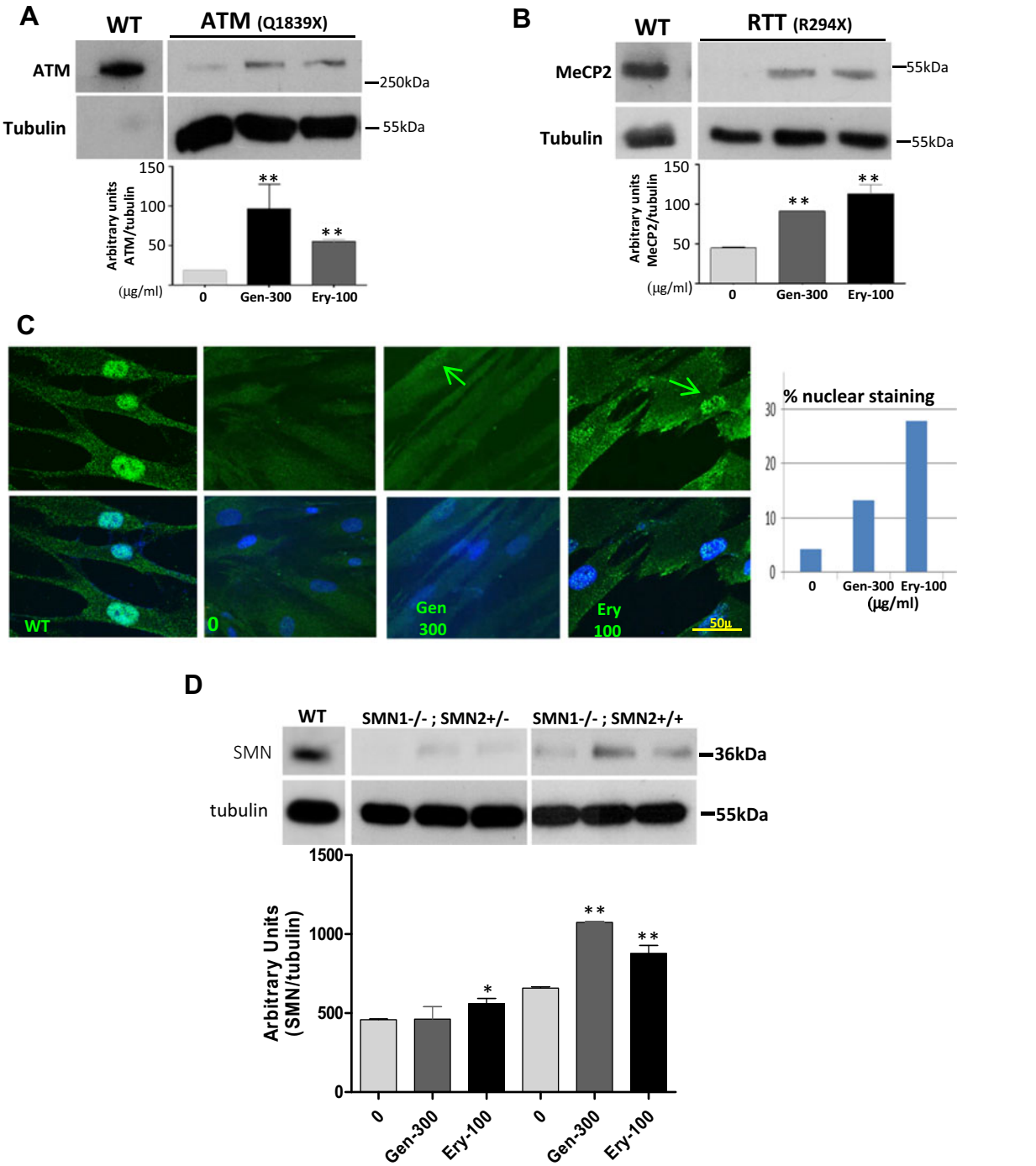


Fig. 3 Antibiotic-induced read-through in patient-derived cells. **a** β-Lymphocytes from A-T patients carrying a nonsense mutation in position 5515 ATM(Q1839X) were treated with the indicated antibiotics for 7 days. Cells were then harvested and analyzed by WB using a specific anti-ATM antibody. **b** Fibroblasts from RTT patients carrying the stop-codon R294X were treated as in **a**. Cells were then analyzed as above using a specific anti-MeCP2 antibody. **c** IF staining of WT and treated fibroblasts using the specific anti-MeCP2 antibody (*green*) and DAPI for nuclear staining (*blue*). *Bars* (*right panel*) represent percent of nuclear staining counted in five fields (10–30 cells per field). **d** Fibroblasts harvested from SMA patients SMN1−/−, SMN2+/−, or SMN2+/+ were treated as in **a**. Cells were then analyzed using a specific anti-SMN antibody. Band intensity analyses (using TINA software) are presented in the *lower panels*. *Ery-100* erythromycin 100 μg/ml, *Gen-300* gentamycin 300 μg/ml. Values are shown as mean±SD from at least three independent experiments. *Asterisks* denote statistical significance of treated samples as compared to untreated samples in an unpaired Student's *t* test. *$P \leq 0.05$; **$P \leq 0.01$

anti-MeCP2 antibody. Our results show that MeCP2 was expressed only in the presence of the antibiotics, in comparison to non-treated cells (Fig. 3b). Next, we examined the subcellular localization of MeCP2. As MeCP2 is a nuclear

protein that binds methyl CpGs in DNA to modulate transcription, it is considered active when appropriately sequestered to the cell nucleus [44]. Indeed, following treatment, there is evidence for nuclear localization of the MeCP2 protein (Fig. 3c). The percentage of cells exhibiting nuclear MeCP2 staining before and after treatment is shown in the right panel.

Spinal muscular atrophy (SMA) is an inherited neurodegenerative disease caused by mutations in the survival motor neuron 1 (SMN1) gene [45]. Most SMA cases arise from SMN1 deletions [45]. SMN2 is nearly identical to SMN1; however, most SMN2 transcripts are unstable. A downstream SMN2 stop codon is an excellent candidate for drug-induced PTC read-through as data show that the addition of a C′-terminus "tail" greatly induces the SMN2 protein stability. SMN1-deficient fibroblasts [containing either one (+/−) or two (+/+) copies of the SMN2 gene (Coriell: #GM00232 and #GM0967, respectively)] were treated for 7 days with the indicated antibiotics. Following drug treatment, the cells were harvested and subjected to Western blot analysis. As demonstrated in Fig. 3d, the full-length SMN protein was expressed in both types of fibroblasts. In addition, expression was reduced when one allele of the SMN2 gene was also mutated. Although restoration treatment leads to low expression of wild-type protein, in some cases, these levels may be sufficient to ameliorate the disease symptoms [14, 15].

Antibiotic-induced read-through of APC mutations

Colorectal cancer (CRC) is one of the most common cancer types. Adenomatous polyposis coli (APC) is mutated in most CRC patients [46]. APC's critical role in tumorigenesis lies in its ability to negatively regulate β-catenin nuclear levels [47]. Around 30 % of the APC mutations are premature stop codons. SW1417 is a CRC cell line that expresses an APC protein harboring a stop codon at position 1450 [48]. In the following experiment, cells were treated for 7 days with gentamycin or erythromycin (100 μg/ml) and then harvested and analyzed by Western

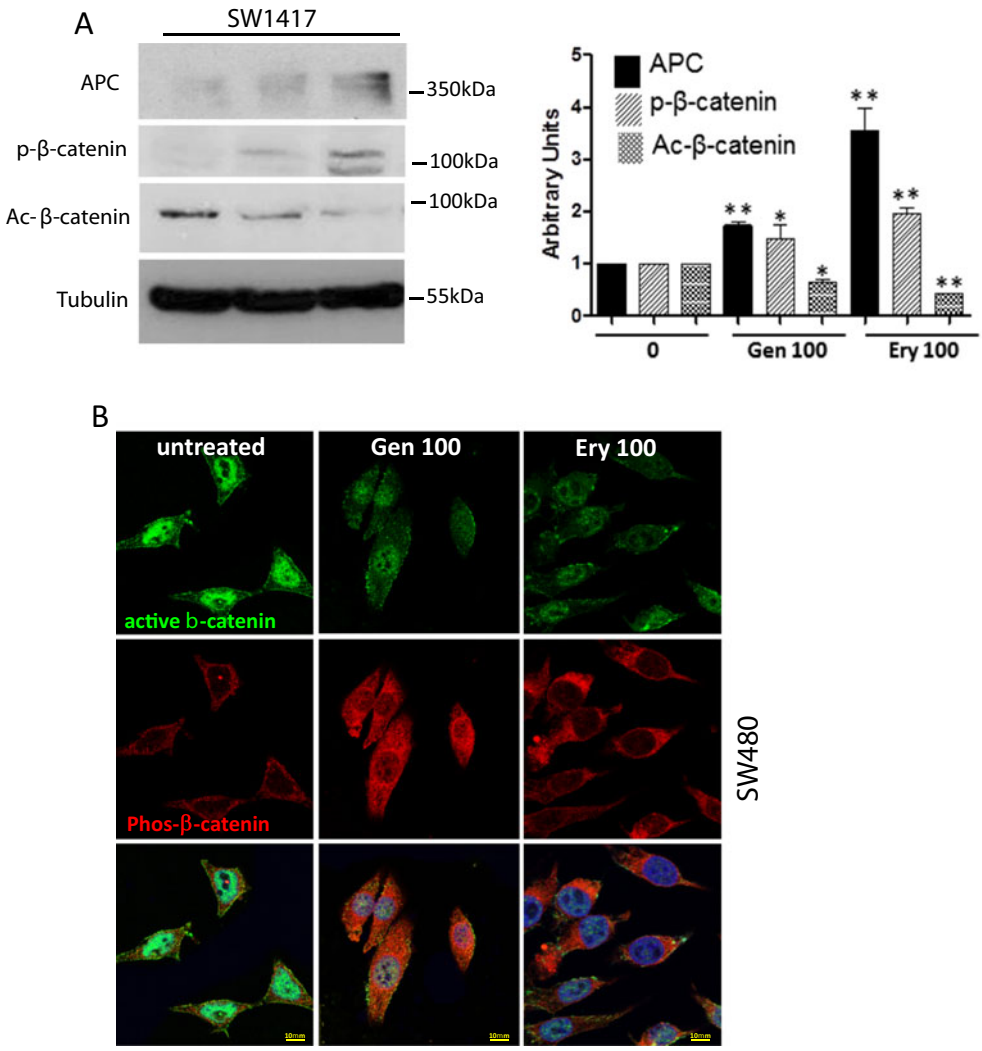


Fig. 4 Erythromycin effect on restoration of APC levels and function in CRC cell lines. Colon carcinoma cell lines SW480 and SW1417 were treated as indicated for 7 days. **a** SW1471 cells were harvested and separated by SDS-PAGE. Blots were incubated with anti-APC, anti-phospho-, or anti-active-β-catenin antibodies. Band intensity analysis (using TINA software) is presented in the *right panel*. **b** SW480 cells seeded on cover slips were treated as described above. Cells were fixed, permeabilized, incubated with anti-active- or anti-phospho-β-catenin antibodies, and viewed using confocal microscopy. Cell nuclei were stained with DRAQ5 reagent. *Ery-100* erythromycin 100 μg/ml, *Gen-300* gentamycin 300 μg/ml. Values are shown as mean±SD from three independent experiments. *Asterisks* denote statistical significance of treated samples as compared to untreated samples in an unpaired Student's *t* test. **P*≤0.05; ***P*≤0.01

blotting. As shown in Fig. 4a, there is an increase in the expression levels of both APC and phospho-β-catenin (the β-catenin form that is marked for degradation). In addition, the active form of β-catenin that induces Wnt signaling is significantly reduced. The effect of these antibiotics was also examined, using immunofluorescence (IF), in SW480 cells that contain a stop codon at position 1338 of the APC gene [48]. The antibiotic-treated cells were fixed, permeabilized, and incubated with anti-active β-catenin (the active form that localizes to the nucleus) and anti-phospho-β-catenin antibodies. The results show that treatment led to reduced levels of nuclear active β-catenin and increased levels of cytoplasmic phospho-β-catenin (Fig. 4b). Taken together, these results indicate

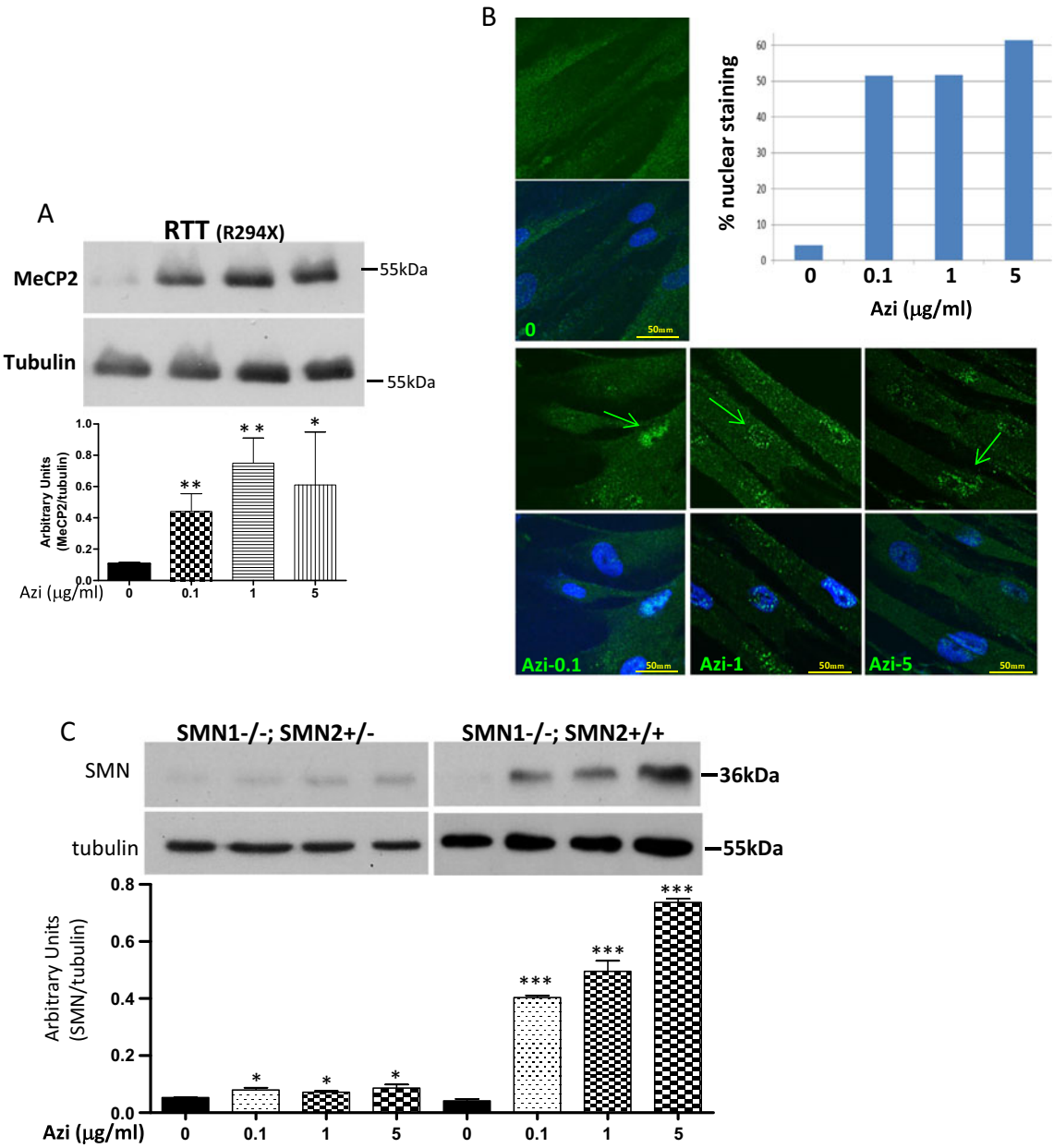


Fig. 5 Azithromycin-induced read-through in patient-derived cells. **a** Fibroblasts from RTT patients carrying the stop-codon R294X were treated with azithromycin for 7 days. Cells were then harvested and analyzed using a specific anti-MeCP2 antibody. Band intensity analysis (using TINA software) is presented in the *lower panel*. **b** IF staining of WT and treated fibroblasts using the specific anti-MeCP2 antibody (*green*) and DAPI for nuclear staining (*blue*). *Bars* (*right panel*) represent percent of nuclear staining counted in five fields (10–30 cells per field). **c** Fibroblasts from SMA patients SMN1−/−, SMN2+/−, or SMN2+/+ were treated with azithromycin for 7 days. Cells were then harvested and analyzed using a specific anti-SMN antibody. Band intensity analysis (using TINA software) is presented in the *lower panel*. *Azi* azithromycin. Values are shown as mean±SD from at least three independent experiments. *Asterisks* denote statistical significance of treated samples as compared to untreated samples in an unpaired Student's *t* test. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.008$

that, in both cases, the restored APC protein re-gained its function as a scaffolding component of the β-catenin-destruction complex.

Azithromycin-induced read-through in patient-derived cells

The erythromycin derivative azithromycin has a longer half-life than erythromycin and is also currently in clinical use. Our preliminary experiments revealed that azithromycin can induce stop-codon read-through at low concentrations (not shown). Indeed, a dose-response analysis of azithromycin demonstrated high expression levels of mutated proteins even at a low dose of 0.1 μg/ml. Figure 5a, c depicts the results from several experiments performed in RTT and SMA fibroblasts. IF analysis demonstrates that the restored MeCP2 protein is localized to the nucleus (as expected) in 50–60 % of the cells (Fig. 5b). This observation suggests that azithromycin can induce PTC read-through and, importantly, that azithromycin can be used at relatively low concentrations which is of great importance in the case of chronic usage as needed from a drug that is expected to induce PTC read-through.

Erythromycin treatment reduces intestinal polyp number

$Apc^{Min/+}$ mice carry a nonsense mutation in the APC gene at codon 850 that leads to the expression of a truncated, non-functional APC product [49]. As a result, these mice develop multiple intestinal polyps. In the following experiment, the intestines of age-matched (12 weeks) untreated and erythromycin-treated $Apc^{Min/+}$ mice were examined for polyp incidence. Our data shows that erythromycin treatment for 4 months led to a significant reduction in the total number of polyps (Fig. 6a, b) as observed by gross analyses of the entire intestinal mucosa stained with 0.5 % methylene blue. In addition, total RNA was extracted from intestines of untreated and erythromycin-treated mice. The Illumina Direct Hyb Assay array results show that a total of 654 genes were differently expressed with cutoff p value <0.05 and fold-change difference >|5|. These include genes such as EFG and CD3E that are known to be modified by Wnt signaling. Function enrichment of the 199 (30 %) upregulated and the 455 (70 %) downregulated genes is shown in Table 2.

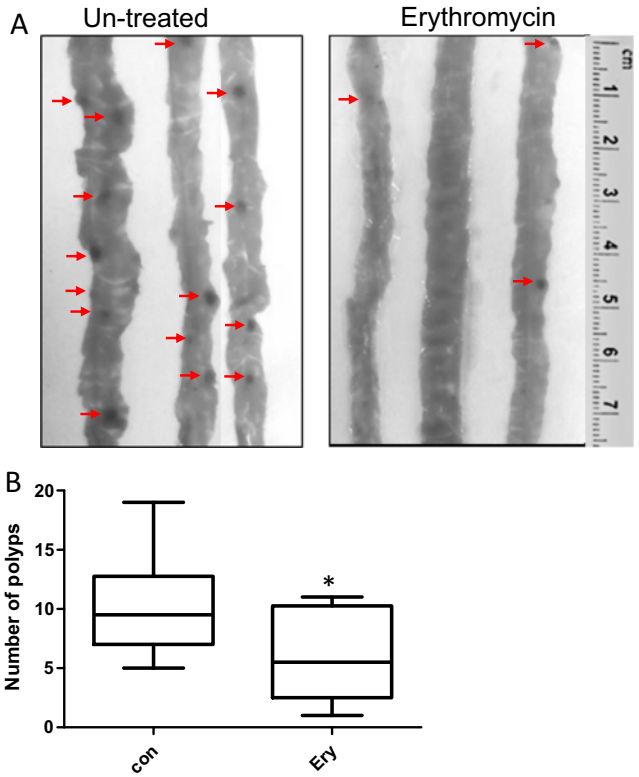


Fig. 6 Erythromycin effect on $APC^{Min/+}$ mice intestine polyps. **a** Representative macroscopic view of intestines isolated from age-matched (21-week-old) $Apc^{Min/+}$ mice that were either untreated or injected with 600 μg erythromycin 5 days a week for a total of 16 weeks. Specimens were stained with 0.5 % methylene blue. Ten mice per group were used for each treatment. **b** Statistical analysis of polyp number throughout the entire intestine (n=10). *P<0.05, Student's t test

Discussion

More than 1800 inherited human diseases are a direct outcome of alterations within the genetic code that result in prematurely arrested translation leading to the production of truncated non-functional proteins. For over three decades, it has been accepted that the effect of many of these nonsense mutations can be partially alleviated by non-traditional read-through agent treatment. It has been shown that members of the aminoglycoside and macrolide antibiotic family, as well as other molecules such as PTC124, can produce these beneficial effects [17]. Even though protein translation is typically restored at only a low capacity (rendering ≤5 % correction), drug treatment can still improve protein function. In an effort to expand upon these promising results with respect to antibiotic-induced PTC read-through, we developed a novel screening vector based on fluoro-proteins. This vector is superior to the luciferase-based construct as the latter may be targeted by some read-through agents [19]. The common assumption is that the sequence context within the mRNA influences the translation apparatus in response to subtle changes mediated by aminoglycosides within the ribosomal decoding site [11]. It has also been reported that the sequence surrounding the PTC plays an important role in determining its susceptibility to aminoglycoside-mediated read-through. Several studies presented extensive comparison between the different stop codons and adjacent nucleotides [11, 25, 50–58] and concluded

Table 2 Function enrichment of the 199 (30 %) upregulated and the 455 (70 %) downregulated genes

Functional enrichment of genes upregulated		Functional enrichment of genes downregulated	
Function category	Genes	Function category	Genes
Immune response	IGHG, TNFSF10, IL18, OASL1, TNFRSF17, CFI, MX2, CD3E	Cytoskeleton (mostly actin) binding proteins	MYH1, MYH2, MYH4, ACTN2, MYBPC2, TNNC1, SYNPO2, ACTN3, TPM2, FLNC, CAPZB, MYOT, TNNI2, PKNOX2, PFN2, NRAP, XIRP2, MYPN, LIMCH1, SPNB1, ABRA, TMOD1, TMOD4, PACSIN3, LMOD3, TRIM54, MTAP4, ULK2, EGF
Defense response	REG3B, IGHG, SAA2, SAA1, CFI, NOS2, REG3G, MX2	Cell adhesion (mostly tight junctions)	ACTN2, ACTN3, AMOTL1, PRKCQ, MYL2, MYH1, MYH2, MYH4, MYLPF, NRAP, XIRP2, RAPSN, NEURL2, CHRNA1, SYNGR1, CAPZB, CAMK2A, CHRNA10, ABCB4, CDH13, CDH15, MYBPC2, ATP1B2, CNTNAP2, VTN
Plasma membrane components	ITGAE, UGT1A6A, ABCC3, SLCO2A1, CD3E	Focal adhesion	CAV3, MYL2, MYLK2, MYLPF, ACTN2, VTN, ACTN3, EGF, FLNC
Regulation of apoptosis	MOAP1, CD3E, IL18, PIM1	Negative regulation of apoptosis	HSPA1L, NOL3, EEF1A2, PHLDA3, ADORA1
Regulation of gene expression and transcription	EIF4EBP3, BARX2, IRF1, THAP1, IGHG, UBD, CFI	Negative regulation of transcription	MYF6, LMCD1, NFIC, PKIA, ADORA1, HHATL
Cell differentiation	BARX2, CD3E, SOCS3, IRF1, UBD, HEPH, DMBT1	Cell differentiation	MEF2C, IRX5, MYL2, TCAP, FHL1, TTN, MLF1, HSPA1L, ANK1, SMARCD3, NDRG2, CHRNA1, ACSL6, UNC45B, MB, MYF6, SRPK3, ACTC1, ACTA1, TBX1, KY, FLNC, CACNA1S, CSRP3, CAPN3, LPIN1, TACC2, HOXC10, TRIM54, KRT17, ULK2, SIX1, KRT14, VAMP5, BIN1, NEURL2, TMOD1
Signal transduction	PLCB3, SOCS3, OLFR1418, NOS2	Signaling pathways	AK1, MAPK1IP1, TPD52L1, CAMK2A, TACC2, MLF1, MEF2C,EGF,HSPB, PRKAG3, PPP1R3C, PYGM, PHKG1, PRKAB2, PHKA1, ACACB, MYL2, TNNC2, EPDR1, MYL3, TNNC1, MYL1, SRL, TTN, PVALB, PLCD4, DTNA, CACNA2D1, CDH15, CAPN13, ITGB1BP2, ATP2A1, RYR1, ALOX5, CASQ1, SGCA,
		Muscle cell development and differentiation	ACTC1, ACTA1, TCAP, MYL2, KY, FLNC, TTN, CACNA1S, CAPN3, MYO18B, CHRNA1, NEURL2, TMOD1

Functional enrichment analysis was performed using DAVID tool. Differentially expressed genes with cutoff fold-change >5 and $P<0.05$ were selected for analysis. A total of 554 genes were obtained, 199 were upregulated, and 455 were downregulated when treatment was compared to control

that the identity of the nucleotide immediately downstream of the stop codon (+4, with the first nucleotide of the termination codon as +1) strongly influences the termination-signal strength. Yet, there is a large diversity in the sequences needed for the activity of read-through inducing agents. For example, it is acceptable that the UAA codon is the strongest stop codon [56, 59]; however, some UAA codons have higher read-through levels than some UGA or UAG codons [12]. It seems that the sequence context surrounding the stop codon only somewhat influences the efficiency of read-through. Although there are dissimilarities among different groups regarding the ability of various compounds to induce read-through, and on the nature of the sequence that is most susceptible to read-through, the fact that slight recovery of protein levels could be enough to cure or ameliorate the symptoms of some devastating diseases is sufficient to encourage further discussion and research concerning the development of the most efficient read-through agent. Our results, using the new vector, show random influence of stop codon type or context on read-through efficiency. Therefore, no complete consensus stop codon type or adjacent sequence is implied to predict the best outcome. This can be the result of other surrounding sequences and the various different drug compounds, concentrations, duration of treatment, and cells used for nonsense suppression. We believe that our reporter system is an effective tool for examining a large number of different sequences and compounds in order to address this issue.

Approximately one third of all inherited genetic diseases are caused by premature stop codons. The incidence of nonsense mutations in individual cases of genetic disorders can range from 5 to 70 % [17]. Here, we show proof-of-concept for the ability of macrolide antibiotics to read-through PTCs

that are known to be the cause of several diseases, such as mutations in the ATM gene in ataxia-telangiectasia (A-T), MeCP2 that result in RTT syndrome, the SMN gene in the SMA syndrome, and mutations in the adenomatous polyposis coli gene that lead to familial adenomatous polyposis (FAP). Our data relies upon experimental procedures in both reporter assays and mammalian cells harboring the disease-causing mutations. Results show that erythromycin and the newer antibiotic azithromycin can induce PTC read-through. Importantly, azithromycin can induce read-through at a 100-fold lower concentration than erythromycin or gentamycin. Reducing dosage levels is extremely important from a clinical perspective, as many of these antibiotics may have significant adverse side effects upon long-term treatment of patients.

The $Apc^{Min/+}$ mouse phenotype resembles the human disorder FAP, and we have previously shown that this mouse is a suitable in vivo pre-clinical model for examining the effects of APC gene nonsense mutation read-through [13]. The present study is consistent with our previously published results (with the macrolide antibiotic, tylosin [13]), showing that erythromycin treatment can also reduce polyp number. In addition, we have identified widespread changes in gene expression patterns among a broad array of cellular functions. Most genes were down-regulated (455 out of 654), and among these are many that are known to be tightly involved in the Wnt signaling cascade, such as EGF [60]. Other genes are indicated in cell adhesion, such as different members of the actin and cadherin protein families. Although the mechanism of how macrolide induce stop-codon read-through is far from understood, our data suggest that macrolides may prove to be effective read-through agents in different PTC-mediated diseases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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