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THE Mg^{2+} TRANSPORTER MagT1 PARTIALLY RESCUES CELL-GROWTH AND Mg^{2+} UPTAKE IN CELLS LACKING THE CHANNEL-KINASE TRPM7

Francina Deason-Towne[a], **Anne-Laure Perraud**[a], and **Carsten Schmitz**[a]
[a]Integrated Department of Immunology, National Jewish Health and University of Colorado Denver, Colorado, 80206, USA

Abstract

Magnesium (Mg^{2+}) transport across membranes plays an essential role in cellular growth and survival. TRPM7 is the unique fusion of a Mg^{2+} permeable pore with an active cytosolic kinase domain, and is considered a master regulator of cellular Mg^{2+} homeostasis. We previously found that the genetic deletion of TRPM7 in DT40 B-cells results in Mg^{2+} deficiency and severe growth impairment, which can be rescued by supplementation with excess extracellular Mg^{2+}. Here, we show that gene expression of the Mg^{2+} selective transporter MagT1 is upregulated in TRPM7$^{-/-}$ cells. Furthermore, overexpression of MagT1 in TRPM7$^{-/-}$ cells augments their capacity to uptake Mg^{2+}, and improves their growth behavior in the absence of excess Mg^{2+}.

Keywords

MagT1; Mg^{2+} homeostasis; channel-kinases; TRPM7 complementation; DT40 B cells

1. Introduction

As the most abundant intracellular divalent cation, Mg^{2+} is essential for a multitude of physiological processes, including protein synthesis and cell proliferation [1,2]. Transport of Mg^{2+} across membranes plays an important role in maintaining cellular equilibrium [3], yet the molecular mechanisms underlying vertebrate Mg^{2+} homeostasis have just begun to be characterized [4,5]. In this study we focus on the ability of one Mg^{2+} transporter, MagT1 (Magnesium transporter subtype 1), to complement the deletion of TRPM7 (Transient Receptor Potential Melastatin 7), an ion channel shown to play a major role in regulating cellular Mg^{2+} homeostasis [5,6].

TRPM7 and its closest relative, TRPM6, are the only examples of ion channels covalently linked to a kinase domain. Biophysical studies have shown that TRPM7 is a channel selective for divalent cations and can be inhibited by intracellular Mg^{2+} or MgATP [7]. The genetic disruption of TRPM7 in DT40 B-cells results in reduced intracellular Mg^{2+} levels

Address correspondence to: Anne-Laure Perraud, PhD, and Carsten Schmitz, MD, Integrated Department of Immunology, National Jewish Health, 1400 Jackson St, Denver, CO 80206, USA. Phone: (303) 270 2078; Fax: (303) 270 2325; perrauda@njhealth.org, schmitzc@njhealth.org.

and cell growth arrest under physiological concentrations of Mg^{2+} (0.5–1 mM). Importantly, this phenotype can be reversed by supplementation with high (10 mM) extracellular Mg^{2+}, but not by adding Ca^{2+}, Mn^{2+}, Zn^{2+}, or Ni^{2+} to the culture media [8]. Similarly, patients deficient in TRPM6 suffer from lethal seizures, but can live normally if nutritionally supplemented with Mg^{2+} [9,10]. Others have demonstrated that in *C. elegans* and zebrafish, TRPM7 homologues are also involved in Mg^{2+} regulation [11,12]. TRPM7^{-} deficiency in mice is embryonic lethal, though rescue by supplemental Mg^{2+} was not tested in this study [13]. Recently, a different group published that embryonic stem cells lacking TRPM7 kinase show minimal TRPM7 channel activity and a proliferation arrest phenotype that, similar to the TRPM7-deficient DT40 cells, can be rescued by Mg^{2+} supplementation [14].

Currently, the only known selective plasma membrane Mg^{2+} transporter is MagT1. It has been demonstrated to be essential for vertebrate Mg^{2+} influx [3], and is broadly expressed in human tissues [15]. It was originally discovered as an upregulated transcript in mouse distal convoluted tubule (MDCT) cells grown under hypomagnesic conditions [16]. MagT1 was also recently captured in a screen designed to identify human molecules that can complement for the deficiency in the Alr1p Mg^{2+}-transporter in yeast [15]. Knockdown of MagT1 leads to early developmental arrest in zebrafish embryos, and excess supplemental Mg^{2+} was shown to rescue this developmental block [15]. These studies and others also revealed that varying concentrations of extracellular Mg^{2+} can regulate expression of MagT1 in cell lines and murine tissues [17,18].

Given these reports, we asked whether MagT1 levels are increased in TRPM7$^{-/-}$ DT40 cells, possibly to help compensate for the negative effect of TRPM7-deficiency on intracellular [Mg^{2+}], and show here that this is indeed the case. Furthermore, we find that overexpression of recombinant MagT1 in TRPM7$^{-/-}$ cells partially rescues the ability of these cells to uptake Mg^{2+}, as well as their growth-defect in the absence of Mg^{2+} supplementation. Our results lend further credentials to the notion that MagT1 is a potent Mg^{2+} transporter pathway in vertebrates.

2. Materials and Methods

2.1. Molecular Biology

The coding sequence of chicken *MagT1* was extracted from DT40 B cell cDNA using oligos designed from the *gallus gallus MagT1* published sequence (GenBank ID: NM_001006435) 5’- ACGTGGTACCACTCATTAGGAAACTGTATGGATATCC -3’ and 5’- ACGTAAGCTTATGGCGGCGCTGCCGGTACTTGTG -3’. Chicken *MagT1* was then cloned into the pcDNA4/TO-C-term-FLAG expression vector (KpnI/HindIII), allowing the expression of the C-terminally FLAG-tagged transporter.

2.2. DT40 Cell Line Construction, Cell Culture, and Growth Curves

The generation of DT40 TRPM7$^{-/-}$ cells, the cloning of human TRPM7 WT, and the establishment of stable inducible expression of hTRPM7 in TRPM7$^{-/-}$ DT40 cells have been previously described [8]. TRPM7$^{-/-}$ DT40 cells expressing the tet –repressor were transfected with pcDNA4/TO-constructs encoding C-terminally FLAG-tagged chicken *MagT1*. Cells were maintained in chemically defined HyQ CCM1 media supplemented with 10 mM $MgCl_2$, P/S, Glu, 1% chicken serum, and 1mg/ml Zeocin (Invitrogen), 50 μg/ml Blasticidin (Invivogen). Growth curves were generated using customized Mg^{2+} free CCM1 media supplemented with 0.5 mM $MgCl_2$ and 1% chicken serum.

2.3. Real-time Quantitative PCR analyses

DT40 WT and TRPM7$^{-/-}$ cells were cultured in Mg^{2+} deficient media for 0, 4, or 24 hours. Total RNA was isolated from cells using RNeasy (Qiagen) and converted to cDNA using Superscript III RT (Invitrogen). Quantitative RT-PCR was carried out using Sybr-Green Master Mix and DNA Engine Opticon 2 (BioRad formerly MJ Research). Primers used for detection of *MagT1* (For 5'- GTGAACTATATCCATGGAAGC -3' and Rev 5'-TCCTAAAGTAACACCACCATTG -3') and *GAPDH* control (For 5'-TTGTTTCCTGGTATGACAATGAGTTT-3', Rev 5'-CTCACTCCTTGGATGCCATGT -3').

2.4. Immunoblotting

Stable cell lines expressing MagT1 were lysed in 0.5% Triton lysis buffer, and proteins immunoprecipitated using anti-Flag M2 agarose beads at 4°C overnight under constant rotation. Beads were washed 3× with lysis buffer and proteins were eluted by boiling in 2× sample buffer. Proteins were separated by SDS-PAGE using 12.5% polyacrylamide gels, transferred to a polyvinylidene difluoride membrane, and analyzed by anti-FLAG (Sigma) immunoblotting.

2.5. Mg^{2+} Measurements

Intracellular concentrations of Mg^{2+} were measured using a QM-6/2003 spectrofluorometer (Photon Technology International). As previously described [19], cells were grown in chemically defined media supplemented with 10 mM $MgCl_2$ and when required for induction of gene expression, treated with doxicycline 48 hours before being placed in 0.5 mM $MgCl_2$. At 14–18 hours, 4×10^6 cells per sample were loaded with 1μg/ml Mag-Fura-2 (Invitrogen) for 30 min at room temperature in 0.5 mM $MgCl_2$ buffer (135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5.6 mM Glucose, 10 mM Hepes, adjust pH to 7.5, then add 0.1% bovine serum albumin). The cells were washed, placed in a cuvette with a stirbar, and measured every 2 s at 340/380 nm excitation accompanied by monitoring 510 nm emission light for 10 min, with 9.5 mM or 19.5 mM of $MgCl_2$ added directly into the cuvette at t=40s. The area under the curve was calculated by averaging the fluorescence-ratio (F340/F380) of the first 39s (before $MgCl_2$ addition) to obtain baseline average. The baseline was subtracted from each timepoint in the indicated response (40–600s) and these values were summed and normalized to TRPM7$^{-/-}$. The SEM was calculated for each group and a two-tailed t-test performed on four separate experiments.

3. Results

3.1. TRPM7$^{-/-}$ DT40 cells show higher MagT1 gene expression levels than wildtype cells

TRPM7$^{-/-}$ DT40 cells become Mg^{2+} deficient and die unless their culture medium is supplemented with 10–20 fold higher $[Mg^{2+}]_e$ than the normal 0.5–1 mM physiologic levels provided to DT40 wildtype (WT) cells. Since it has been previously demonstrated that *MagT1* is upregulated under hypomagnesic conditions, we hypothesized that TRPM7-deficiency might also elicit an increase in *MagT1* gene expression. To test this idea, using quantitative RT-PCR, we analyzed chicken *MagT1* specific transcript levels in WT vs. TRPM7$^{-/-}$ DT40 cells under varying Mg^{2+} levels.

Since TRPM7$^{-/-}$ cells need to be maintained in 10 mM $MgCl_2$ to ensure their survival, we cultured the WT cells under the same conditions for better comparison. As shown in Fig. 1, we detect *MagT1* gene expression in WT DT40 cells at 10 mM $MgCl_2$, and its level is elevated when the cells are cultured in Mg^{2+} free medium for 24hrs ($p< 0.05$). In the same experiment, we also found that *MagT1* gene expression is further increased in TRPM7$^{-/-}$ cells, even in 10 mM $MgCl_2$, but most substantially when TRPM7$^{-/-}$ cells were deprived of

Mg^{2+} ($p<0.01$ and $p<0.001$ respectively, as compared to WT DT40s under the same Mg^{2+} conditions). We therefore conclude from these results that in an effort to compensate for the Mg^{2+} deficiency caused by the absence of TRPM7, TRPM7$^{-/-}$ DT40s are upregulating expression of the Mg^{2+} transporter *MagT1*. Although the added amount of *MagT1* does not appear to be sufficient to rescue growth of the TRPM7$^{-/-}$ cells under normal levels of Mg^{2+}, it might contribute to the rescue of their growth under 10 mM $MgCl_2$.

3.2. Complementation with MagT1 partially restores growth in TRPM7$^{-/-}$ cells under physiologic extracellular [Mg^{2+}]

Based on the observations that TRPM7$^{-/-}$ cells exhibit higher levels of *MagT1*-transcript, we asked the question whether overexpression of recombinant MagT1 would allow TRPM7$^{-/-}$ cells to grow under physiologic concentrations of Mg^{2+} (0.5–1 mM). To this aim, chicken MagT1 cDNA was isolated from WT DT40 cells and cloned into an expression vector that adds a C-terminal Flag-tag for easy detection of the overexpressed protein by immuno-Western blot. We subsequently transfected TRPM7$^{-/-}$ DT40 cells cultured in 10 mM $MgCl_2$ with the MagT1 construct, selected for zeocin-resistant clones, and screened for those with stable expression of flag-tagged MagT1. Two MagT1-expressing clones are shown in Fig. 2A, and clone #2 was used for subsequent experiments.

In order to monitor the growth of the MagT1-overxpressing TRPM7$^{-/-}$ DT40 cells in comparison to TRPM7$^{-/-}$ and WT DT40s, all cell lines were grown in parallel for 6 days in chemically defined media containing either 10 mM or 0.5 mM $MgCl_2$. As anticipated, these cell lines show similar growth behavior under 10 mM Mg^{2+} (not shown to improve graph clarity). However, at 0.5 mM $MgCl_2$, as previously reported, TRPM7$^{-/-}$ cells do not grow [8], whereas growth is partially restored in TRPM7$^{-/-}$ cells overexpressing MagT1, albeit not to WT levels. Thus, overexpression of the MagT1 Mg^{2+} transporter is beneficial to DT40 cells lacking TRPM7, although given the different biological properties of these two molecules, it is perhaps not surprising that the complementation is only partial.

3.3. Overexpression of MagT1 increases intracellular Mg^{2+} recovery in TRPM7$^{-/-}$ cells

To further characterize the functional impact of MagT1-overexpression in TRPM7$^{-/-}$ cells, we wanted to investigate whether MagT1 alters the capacity of TRPM7$^{-/-}$ cells to uptake Mg^{2+}. We therefore performed Mg^{2+} add back experiments in our DT40 cell lines cultured under hypomagnesic conditions, and monitored changes in cytosolic free Mg^{2+} levels by loading the cells with the Mg^{2+} sensitive fluorescent dye Mag-fura, as previously described [19]. All cells were preincubated in 0.5 mM $MgCl_2$ for 14–18 hours, and cytosolic Mg^{2+} was measured after the addition of either 9.5 mM (Fig. 3A), or 19.5 mM $MgCl_2$ (Fig. 3B). Notably, baseline fluorescence ratio in TRPM7$^{-/-}$ cells already appear to reflect lower levels of intracellular free Mg^{2+} than in cells complemented with MagT1 or TRPM7. Following 14–18hrs at 0.5 mM $MgCl_2$, TRPM7$^{-/-}$ cells show the least cytosolic Mg^{2+} recovery when $MgCl_2$ is added, as previously published [8]. When complemented with MagT1, Mg^{2+} uptake is increased in TRPM7$^{-/-}$ cells, and as would be expected, Mg^{2+} uptake is strongest when TRPM7 is reintroduced into these cells. The addition of 19.5 mM instead of 9.5 mM $MgCl_2$ results in similar effects, but more pronounced, indicating that the amplitude of the Mg^{2+} uptake correlates with the amount of added extracellular $MgCl_2$ (Fig. 3B,C). In sum, these results show that the improved growth behavior of MagT1-complemented TRPM7$^{-/-}$ DT40 cells is accompanied by an amelioration of the Mg^{2+} uptake capacity of these cells, suggesting that this is the mechanism by which MagT1 compensates for the absence of TRPM7.

4. Discussion

In this study, we demonstrate that cells lacking the regulator of Mg^{2+} homeostasis, TRPM7, increase gene expression of the Mg^{2+} transporter MagT1, indicating that MagT1 function might be beneficial in situations of TRPM7-deficiency. This notion is further supported by our results showing that overexpression of MagT1 enables TRPM7$^{-/-}$ cells to regain viability and some growth under normal levels of Mg^{2+}. This is in contrast to TRPM7$^{-/-}$ cells that require extracellular Mg^{2+} levels an order of magnitude higher than physiologic concentrations.

Our findings about *MagT1* gene expression regulation are reminiscent of work done in HEK and MDCT cells where *MagT1* expression was also upregulated in response to hypomagnesia [15,16]. Together with our data, these studies suggest that when cells are sensing suboptimal extracellular Mg^{2+}, they respond by transcriptional upregulation of *MagT1*, probably in an effort to avoid intracellular Mg^{2+} deficiency by increasing Mg^{2+} uptake. Importantly, TRPM7 itself is not required to mediate this hypomagnesia-induced *MagT1* upregulation given that this takes place in DT40 cells deprived of TRPM7. Since TRPM7 is described as a master regulator of Mg^{2+} regulation, it could be required to sense environmental Mg^{2+} availability and elicit upregulation of cellular Mg^{2+} transport capacity, but this appears not to be the case for *MagT1*. From our review of the literature, *MagT1* gene expression does not appear to be uniformly regulated in all tissues. Other studies have shown that in low concentrations of Mg^{2+}, mammary epithelial *MagT1* expression remains unchanged [20], while rumen epithelial cells actually downregulate *MagT1* expression [17,18]. These results imply that there may be differential regulation of Mg^{2+} and its transport machinery in various cell types.

We observed that overexpression of MagT1 in TRPM7$^{-/-}$ cells is capable of augmenting growth and increasing cytosolic Mg^{2+} recovery in the absence of supplemental Mg^{2+}. This suggests that MagT1 enhances Mg^{2+} accumulation in TRPM7$^{-/-}$ cells, and supports the role of MagT1 as a Mg^{2+} influx system. These results further establish TRPM7$^{-/-}$ DT40 cells as a valid model system to test functionality of Mg^{2+} transport pathways. This experimental approach has been used previously to demonstrate that SLC41A2, another putative Mg^{2+} transporter, can indeed function as such. Similarly to MagT1, SLC41A2 restores partial growth of the TRPM7$^{-/-}$ cells under normagnesic conditions [21]. In the past, we have also used this system to demonstrate that TRPM7's closest homologue and only other known channel-kinase fusion, TRPM6, cannot functionally compensate for TRPM7 in DT40s [19]. Together, the only partial TRPM7-complementations reported with either SLC41A2 or MagT1 suggest that TRPM7 remains essential for DT40s to proliferate and accrue Mg^{2+} when grown in physiological concentrations of Mg^{2+}. Future studies will need to determine whether overexpression of multiple Mg^{2+} transporters in the absence of TRPM7 can lead to a complete rescue of the growth phenotype without supplemental Mg^{2+}. In summary, our data suggests that MagT1 is an integral component of Mg^{2+} uptake in TRPM7$^{-/-}$ DT40s.

Highlights

- We analyzed the role of the Mg^{2+} transporter MagT1 in DT40 B cells lacking the Mg^{2+} regulator TRPM7.
- Gene expression of MagT1 is upregulated in TRPM7$^{-/-}$ cells.
- Overexpressing MagT1 in TRPM7$^{-/-}$ cells partially rescues their growth in the absence of excess Mg^{2+}.

- Overexpressing MagT1 in TRPM7$^{-/-}$ cells partially rescues their ability to uptake Mg^{2+}.

Acknowledgments

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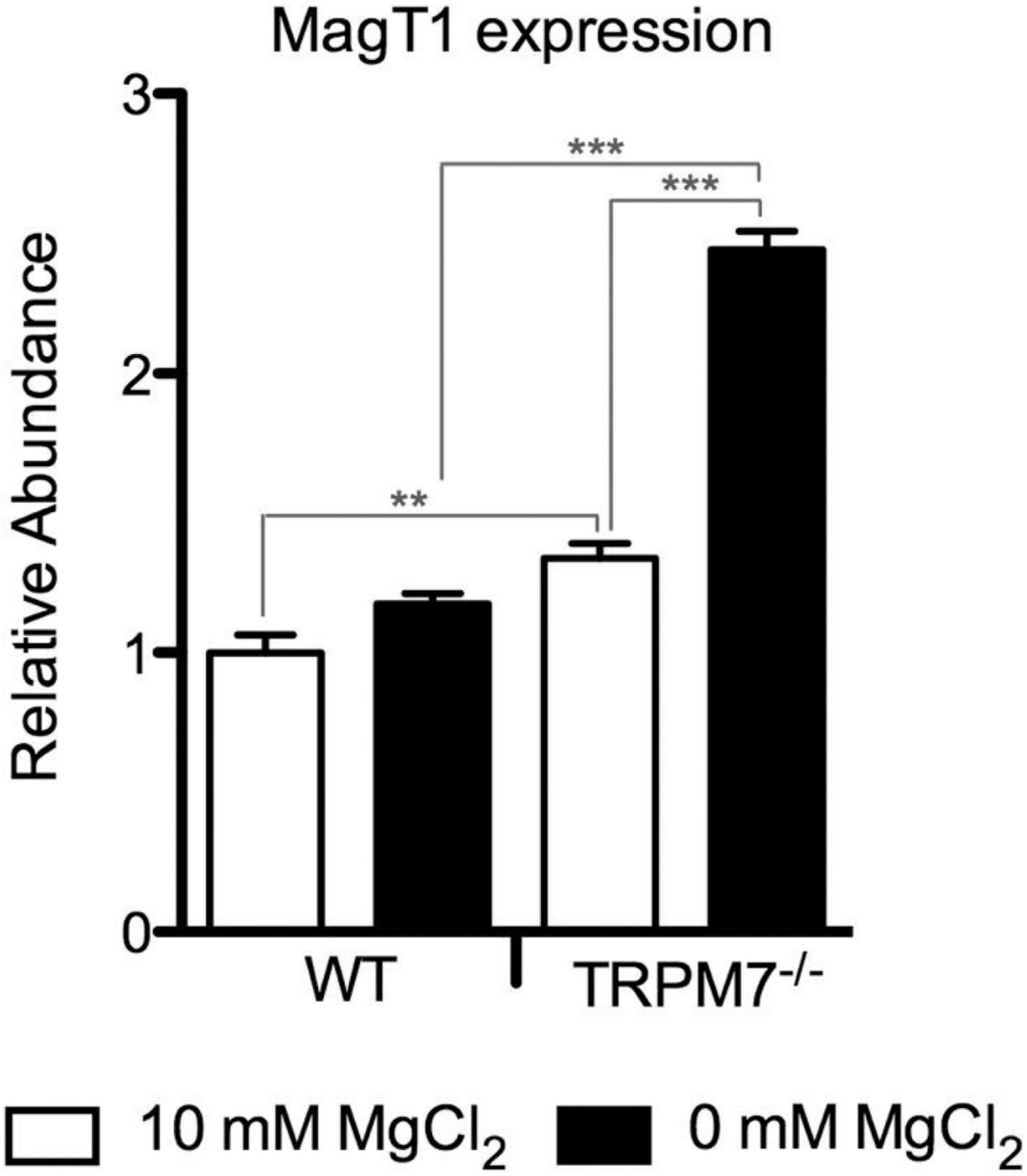

Figure 1. TRPM7$^{-/-}$ DT40 cells show higher *MagT1* gene expression levels than wildtype cells

Quantitative RT-PCR was performed using total RNA from wildtype and TRPM7$^{-/-}$ DT40 cells maintained in media containing either 10 mM Mg^{2+}, or cultured without Mg^{2+} for 24 hours. *GAPDH* levels were used as internal reference. Samples were measured in triplicate with error bars indicating SEM. Graph is from a representative experiment of four. *p < 0.05, **p < 0.01, ***p < 0.001.

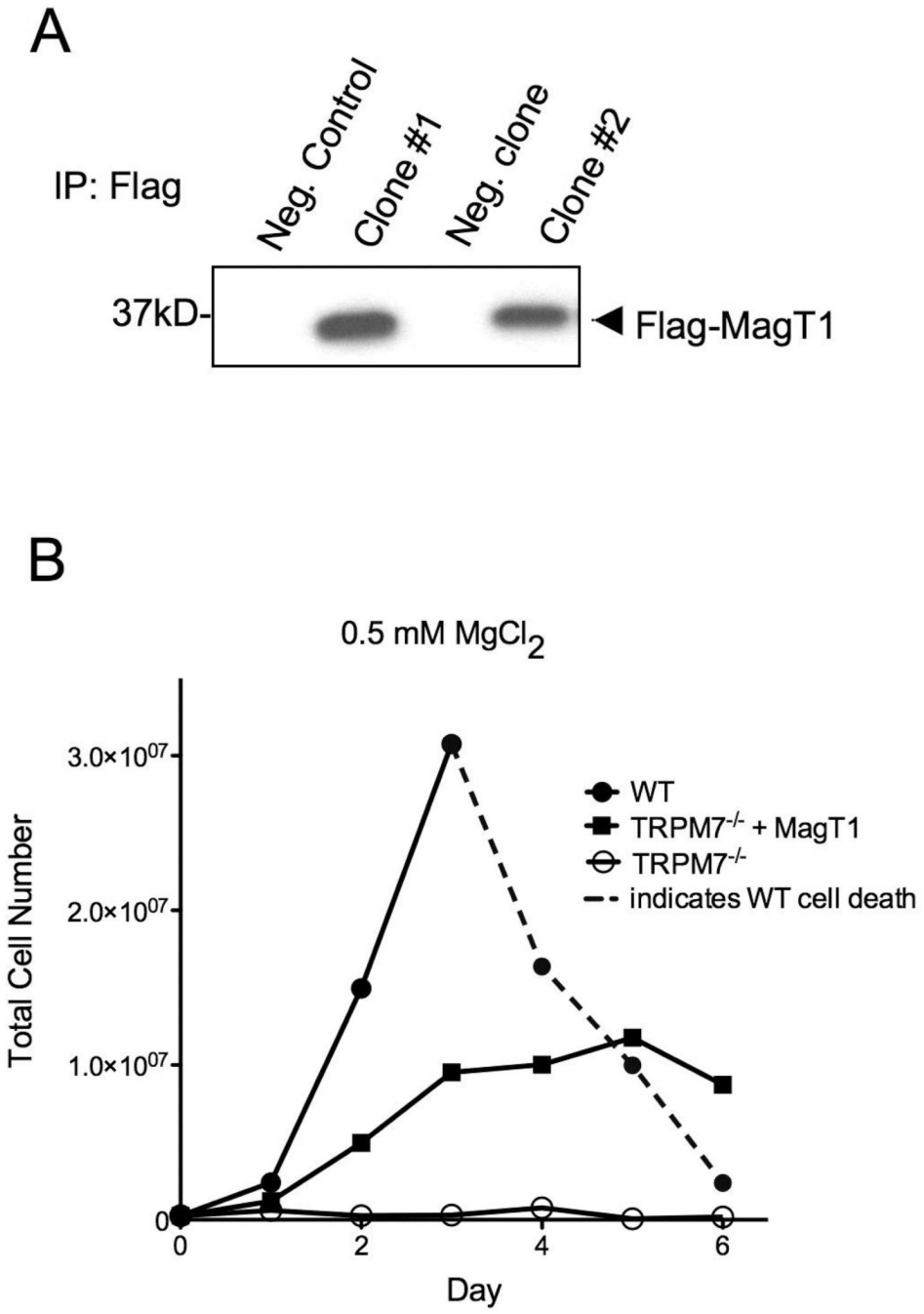

Figure 2. MagT1 overexpression partially reverses growth defect of TRPM7$^{-/-}$ DT40 cells

A) Western Blot showing stable expression of Flag-tagged MagT1 in TRPM7$^{-/-}$ cell clones. Flag-MagT1 was immunoprecipitated using anti-Flag M2-agarose beads, and blot was probed with anti-Flag antibody. TRPM7$^{-/-}$ parental cell line was used as negative control. Positive clone #2 was selected for all subsequent experiments. B) Growth curves recorded in parallel of following DT40 cell lines: Wildtype, TRPM7$^{-/-}$, or TRPM7$^{-/-}$ overexpressing MagT1, all grown in the presence of 0.5 mM Mg^{2+} for 6 days. Dotted line indicates cell death.

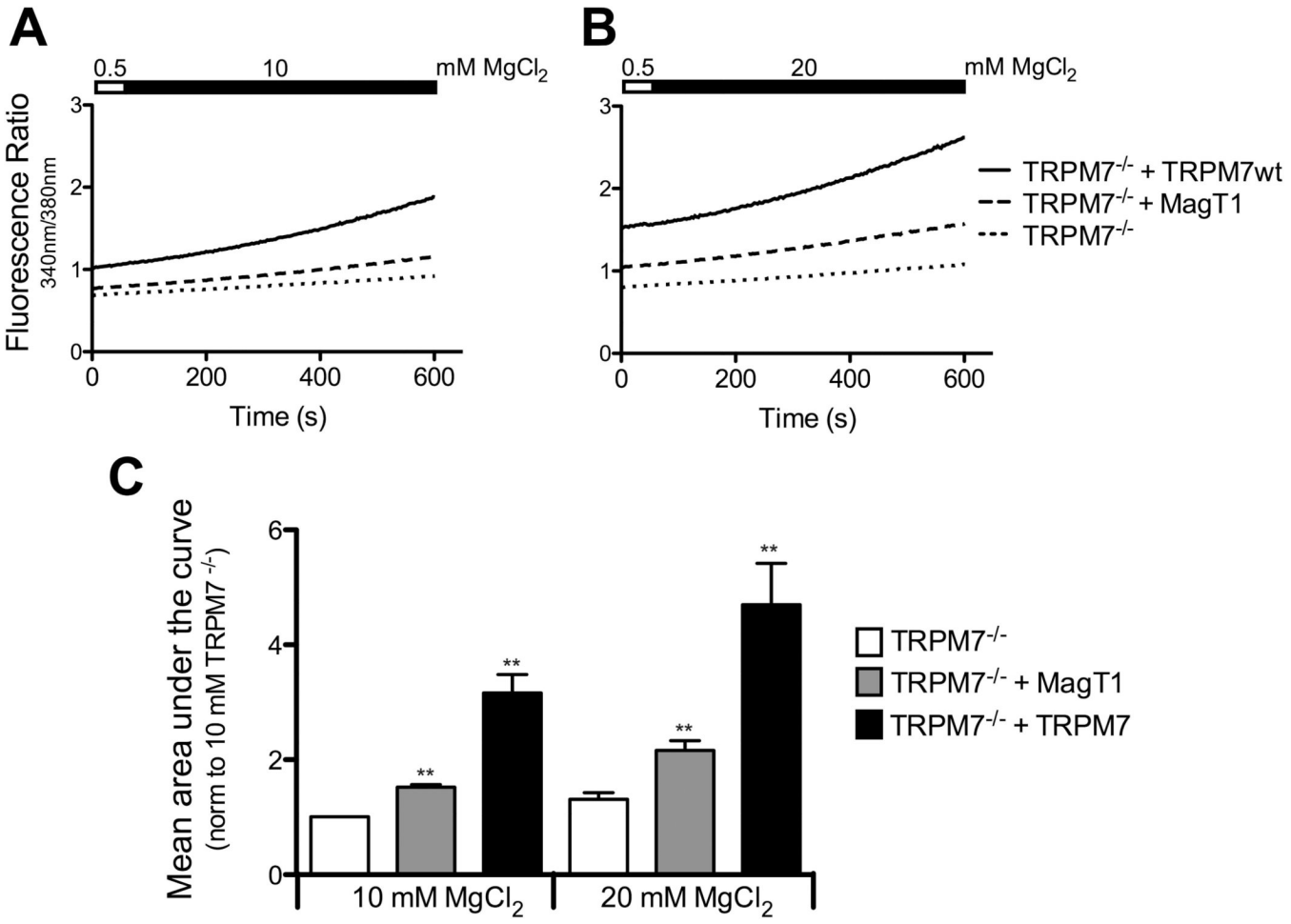

Figure 3. Overexpression of MagT1 increases intracellular Mg^{2+} recovery in $TRPM7^{-/-}$ cells

A) Changes in free cytosolic Mg^{2+} in DT40 wildtype cells in comparison to $TRPM7^{-/-}$ DT40 cells complemented with TRPM7 or MagT1. All cell lines were cultured under hypomagnesic conditions (0.5 mM Mg^{2+}) for 14–18 hours, and then loaded with the Mg^{2+} sensitive fluorescent dye Mag-fura for 30 minutes in a 0.5 mM Mg^{2+} buffer, and samples analyzed using a fluorometer. 9.5 mM $MgCl_2$ were added to the samples as indicated by the black filling in the bar above the graph. Shown trace is representative of four separate experiments. B) Experimental design as in A, except that 19.5 mM $MgCl_2$ were added. Data is representative of six separate experiments. C) Quantification of the results presented under A and B where bars represent mean area under the curve normalized to $TRPM7^{-/-}$ cells at 10 mM final concentration. For statistical analysis, results were compared to values obtained from $TRPM7^{-/-}$ cells at the corresponding $MgCl_2$ concentrations. Data shown are from 4 individual experiments, the mean ± SEM; $^{**}p < 0.01$.