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LeftyA sensitive cytosolic pH regulation and glycolytic flux in Ishikawa human endometrial cancer cells

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ABSTRACT

Objective: LeftyA, a powerful regulator of stemness, embryonic differentiation, and reprogramming of cancer cells, counteracts cell proliferation and tumor growth. Key properties of tumor cells include enhanced glycolytic flux, which is highly sensitive to cytosolic pH and thus requires export of H^+ and lactate. H^+ extrusion is in part accomplished by Na^+/H^+ exchangers, such as NHE1. An effect of LeftyA on transport processes has, however, never been reported. The present study thus explored whether LeftyA modifies regulation of cytosolic pH (pHi) in Ishikawa cells, a well differentiated endometrial carcinoma cell model.

Methods: NHE1 transcript levels were determined by qRT-PCR, NHE1 protein abundance quantified by Western blotting, pH_i estimated utilizing (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein [BCECF] fluorescence, Na^+/H^+ exchanger activity from Na^+ dependent realkalinization after an ammonium pulse, and lactate concentration in the supernatant utilizing an enzymatic assay and subsequent colorimetry.

Results: A 2 h treatment with LeftyA (8 ng/ml) significantly decreased NHE1 transcript levels (by 99.6%), NHE1 protein abundance (by 71%), Na^+/H^+ exchanger activity (by 55%), pHi (from 7.22 ± 0.02 to 7.05 ± 0.02), and lactate release (by 41%).

Conclusions: LeftyA markedly down-regulates NHE1 expression, Na^+/H^+ exchanger activity, pHi, and lactate release in Ishikawa cells. Those effects presumably contribute to cellular reprogramming and growth inhibition.

1. Introduction

LeftyA is a powerful regulator of stemness and embryonic differentiation [1–10]. It suppresses aggressive tumor cell activity [11–14] and is implicated in reprogramming cancer cells [15]. Further, LeftyA activity counteracts cell proliferation, fosters apoptosis and thus inhibits tumor growth [15–18].

Typical properties of cancer cells include strong stimulation of glycolysis with the respective formation of lactate [19,20]. A prerequisite for unhindered glycolytic flux is avoidance of cytosolic acidification, which inhibits the rate limiting enzymes of glycolysis [21]. Mechanisms counteracting cytosolic acidification of tumor cells include activation of Na^+/H^+ exchangers [19,22], Na^+ coupled bicarbonate cotransporters [22] and lactate or monocarboxylate transporters [19,22], extruding both, lactate and H^+ [23].

The present study explored whether LeftyA affects cytosolic pH, Na^+/H^+ exchanger expression and activity as well as glycolytic flux in human endometrial Ishikawa cells.

2. Materials and methods

2.1. Cell culture

Ishikawa cells, a well differentiated endometrial carcinoma cell model [24], were cultured in DMEM/F12 without phenol red media, containing 10% fetal calf serum (FCS), 1% antibiotic/antimycotic solution and 0.25% L-Glutamine (Invitrogen, Karlsruhe, Germany). Cells were treated as described with recombinant human LeftyA (8 ng/ml; 746-LF-025/CF) (R&D Systems, Oxford, UK) and 10 μM Cariporide (Sigma, Munich, Germany).

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2.2. Quantitative real-time PCR

Total RNA was extracted from Ishikawa cultures using Trizol (Invitrogen) based on a phenol-chloroform extraction protocol. Equal amounts of total RNA (2 μg) were reverse transcribed by using the Superscript III First-Strand synthesis system for RT-PCR (Invitrogen) using an oligo dT primer. The resulting first-strand cDNA diluted and used as template in qRT-PCR analysis. L19 was used to normalize for variances in input cDNA. Detection of gene expression was performed with KappaFast −SYBR Green (Peqlab, Germany) and Quantitative RT-PCR was performed on a BioRad iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany). The expression levels of the samples were expressed as arbitrary units defined by the $\Delta\Delta C_T$ method. All measurements were performed in triplicate. Melting curve analysis and agarose gel electrophoresis confirmed amplification specificity.

Primer: NHE1: (Sequence)	forward (5'-3'):ACCTGGTTCATCAACAAGTTCCG reverse (5'-3'): TTCACAGCCAACAGGTCTACCA
Primer: L19: (Sequence)	forward (5'-3'): GCAGCCGGCGCAA reverse (5'-3'): GCGGAAGGGTACAGCCAA

2.3. Western blotting

For determination of NHE1 protein abundance, whole cell protein extracts were prepared by lysing cells in RIPA buffer. Protein yield was quantified using the Bio-Rad DC protein assay kit (Bio-Rad, USA). Equal amounts of proteins (30 μg) were separated on 10% sodium dodecyl sulfate–polyacrylamide (SDS) gel before electrotransfer onto the PVDF membrane (Amersham Biosciences, Germany). Nonspecific binding sites were blocked by overnight incubation with 5% nonfat dry milk in Tris-buffered saline with 1% Tween (TBS-T) (TBS; 130 mmol/L NaCl, 20 mmol/L Tris, pH7.6 and 1% Tween). NHE1 was identified by primary antibodies against human NHE1 (1:500, Sigma, Germany), and antibody against GAPDH (1:1000, Cell Signaling) served as a loading control. For detection, a secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase (HRP) (1:2000, Cell Signaling) or secondary anti-mouse IgG antibody conjugated with HRP (1:2000, GE Healthcare Amersham, UK) was used. Protein complexes were visualized with a chemiluminescent detection kit (GE Healthcare). All experiments were performed in 3 or more cell cultures. Bands were quantified with ImageJ Software.

2.4. Intracellular pH

For digital imaging of cytosolic pH (pH_i), the cells were incubated in a HEPES-buffered Ringer solution containing 10 μM BCECF-AM (Molecular Probes, Leiden, The Netherlands) for 15 min at 37 °C [25]. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epi-fluorescence mode with a 40 x oil immersion objective (Zeiss Neoplan, Germany). BCECF was successively excited at 490/10 nm and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA). Between 10 and 20 cells were outlined and monitored during the course of the measurements. The results from each cell were averaged and taken for final analysis. Intensity ratio (490/440) data were converted into pH_i values using the high-K^+/nigericin calibration technique [26]. To this end, the cells were perfused at the end of each experiment for 5 min with standard high-K^+/nigericin (10 μg/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the r_{max}, r_{min}, pK_a values previously generated from calibration experiments to generate a standard nonlinear curve (pH range 5–8.5).

For acid loading, cells were transiently exposed to a solution containing 20 mM NH_4Cl leading to initial alkalinization of cytosolic pH (pH_i) due to entry of NH_3 and binding of H^+ to form NH_4^+ [25,27]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (β) of the cells [28]. Assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3:

$$ß = \Delta\left[NH_4^+\right]_i \Big/ \Delta pH_i,$$

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[NH_4^+]_i$ is the decrease of cytosolic NH_4^+ concentration, which is identical to the concentration of $[NH_4^+]_i$ immediately before the removal of ammonia. The pK for NH_4^+/NH_3 is 8.9 [29] and at an extracellular pH (pH_o) of 7.4 the NH_4^+ concentration in extracellular fluid ($[NH_4^+]_o$) is 19.37 [20/$(1 + 10^{pHo\text{-}pK})$]. The intracellular NH_4^+ concentration ($[NH_4]_i$) was calculated from:

$$[NH_4]_i = 19.37 \cdot 10^{pHo-pHi}.$$

The calculation of the buffer capacity required that NH_4^+ exits completely. After the initial decline, pH_i indeed showed little further change in the absence of Na^+, indicating that there was no relevant further exit of NH_4^+.

NHE1 activity was measured as the slope of the first 5 min of recovery from acidification and was expressed as ΔpH_i per minute, which could be applied to all measured cells.

The solutions were composed of (in mM): standard Hepes: 115 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4 10 glucose, 32.2 Hepes; sodium free Hepes: 132.8 NMDG Cl, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 KH_2PO_4, 32.2 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl); high K^+ for calibration 105 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 32.2 Hepes, 10 mannitol, 5 μM nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37 °C.

2.5. Lactate concentration in supernatant

Lactate concentration in supernatant were determined utilizing Lactate Assay Kit (Sigma, Germany) an enzymatic assay, which results in a colorimetric (570 nm)/fluorometric ($\lambda_{ex} = 535$ nm/$\lambda_{em} = 587$ nm) value. The kit was used according to the manufacturer's protocol.

2.6. Statistics

Data are provided as arithmetic means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test and only results with $P < 0.05$ were considered statistically significant using Graphpad Software Inc (CA, USA).

3. Results

The present study explored whether LeftyA influences cytosolic pH (pHi) regulation and glycolytic flux in Ishikawa cells.

In a first step, the effect of LeftyA on the transcript levels of Na^+/H^+ exchanger (NHE1) isoform was determined utilizing qRT-

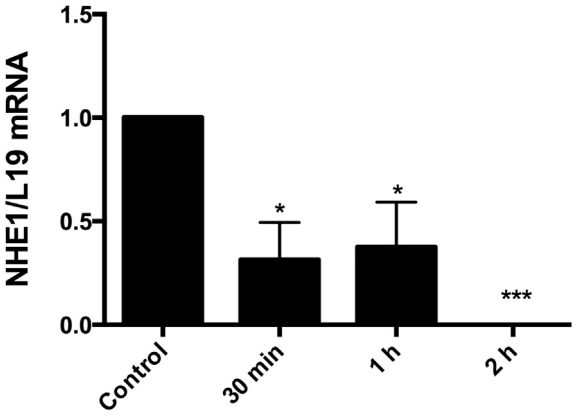

Fig. 1. Effect of LeftyA on NHE1 isoform transcript levels. Arithmetic means ± SEM (n = 4) of NHE1 transcript levels from Ishikawa cells without or with treatment with 8 ng/ml LeftyA lasting upto 2 h. L19 was used as a housekeeping control. * ($P < 0.05$) and *** ($P < 0.0001$) indicates statistically significant difference from untreated cells.

PCR. As illustrated in Fig. 1, Ishikawa cells were treated with LeftyA (8 ng/ml) in a time course lasting 2 h. mRNA was extracted and qRT-PCR was performed. Treatment with LeftyA was followed by a significant decrease of the transcript levels encoding NHE1.

LeftyA similarly influences the protein expression of NHE1 in Ishikawa cells. As illustrated in Fig. 2 A&B, treatment of Ishikawa cells with 8 ng/ml LeftyA for up to 2 h was followed by a significant decline of NHE1 protein abundance.

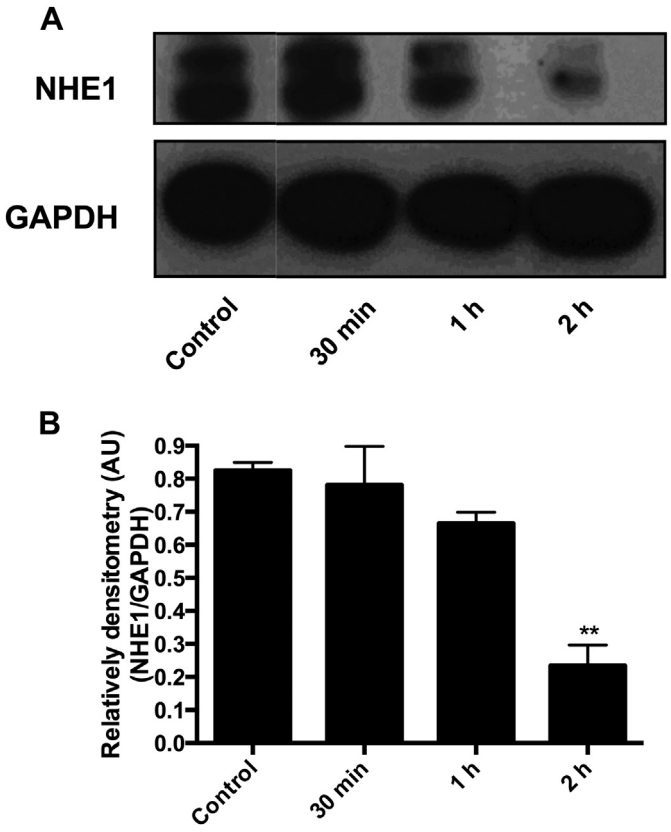

Fig. 2. Effect of LeftyA on NHE1 protein abundance in Ishikawa cells. **A**. Original Western blot of NHE1 and GAPDH protein in cell lysates from Ishikawa cells with or without 2 h treatment with 8 ng/ml LeftyA. GAPDH was used as a loading control. **B**. Arithmetic means ± SEM (n = 3) of the NHE1/GAPDH protein abundance ratios in cell lysate from Ishikawa cells without or following treatment with 8 ng/ml LeftyA. ** ($P < 0.01$) indicates statistically significant difference from untreated cells.

In order to test, whether the down-regulation of NHE1 transcription and protein expression was paralleled by alterations of cytosolic pH (pHi), pHi was estimated from (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein [BCECF] fluorescence.

We explored whether LeftyA modified Na^+/H^+ exchanger activity. To this end, NHE activity was estimated utilizing the ammonium pulse technique (Fig. 3A). Addition of 20 mM NH_4Cl replacing NaCl in the superfusate was followed by NH_3 entry into the cells with subsequent transient cytosolic alkalinization due to binding of H^+ of NH_3 to form NH_4^+ (Fig. 3A). Subsequent removal of NH_4Cl was followed by cytosolic acidification due to NH_3 exit with dissociation of NH_4^+ and cellular retention of H^+ (Fig. 3A). In the absence of Na^+ realkalinization was negligible in both, the absence ($\Delta pH = 0.01 \pm 0.02$ pH/min) and the presence ($\Delta pH = 0.03 \pm 0.02$ pH/min) of LeftyA, an observation pointing to the absence of appreciable Na^+-independent H^+ extruding transport systems. In the absence of LeftyA, the subsequent addition of Na^+ was followed by rapid cytosolic realkalinization, an observation pointing to Na^+/H^+ exchanger activity. As illustrated in Fig. 3B & C, the application of LeftyA was followed by a decline of pHi and NHE1 activity.

The cytosolic acidification following LeftyA treatment was expected to impact on glycolysis. In order to quantify glycolytic flux, lactate concentration was determined in the supernatant of Ishikawa cells following 2 h incubation in the absence or presence of LeftyA (8 ng/ml). As illustrated in Fig. 4, the incubation of Ishikawa cells was followed by a significant increase of lactate concentration in the supernatant, an effect significantly blunted in the presence of LeftyA.

4. Discussion

The present study uncovers a novel and powerful effect of LeftyA on cytosolic pH regulation and glycolytic flux of Ishikawa cells. Treatment with LeftyA is followed by significant decline of NHE1 transcript levels, NHE1 protein abundance, and Na^+/H^+ exchanger activity.

The down-regulation of Na^+/H^+ exchanger activity following LeftyA treatment prevented NHE1 mediated extrusion of H^+ following cytosolic acidification and thus contributed to the maintenance of an acidic cytosolic pHi. Notably, even though Na^+/H^+ exchanger activity is significantly decreased by LeftyA treatment, this effect does not explain the profound cytosolic acidification of the cells following LeftyA treatment. Na^+/H^+ exchanger inhibitor cariporide completely abrogated the realkalinization following ammonia-induced acidification but did not appreciably acidify cells without prior ammonium pulse (data not shown). Na^+/H^+ exchanger activity is highly sensitive to cytosolic pH and is turned off upon cytosolic alkalinization [30]. Apparently, cytosolic pH is in untreated Ishikawa cells high enough to turn off Na^+/H^+ exchanger activity. The cytosolic acidification thus points to dysregulation of an additional H^+ extruding transport protein following LeftyA treatment. The down-regulation of Na^+/H^+ exchanger activity precludes, however, stimulation of Na^+/H^+ exchanger activity following cytosolic acidification and thus plays a permissive role in the down-regulation of cytosolic pH following LeftyA treatment.

As the pK of lactate is below 4 [31], the acid is fully dissociated at the prevailing cytosolic pH and thus glycolysis is paralleled by cellular formation of H^+. In order to accomplish their high rates of glycolysis [19,20] tumor cells up-regulate H^+ extrusion. Cytosolic acidification due to malfunction of Na^+/H^+ exchangers and/or other H^+ extruding transport proteins turns off glycolysis [21]. The sensitivity of glycolysis to cytosolic acidification is an important safeguard of cells [21], as it avoids continued formation of lactate

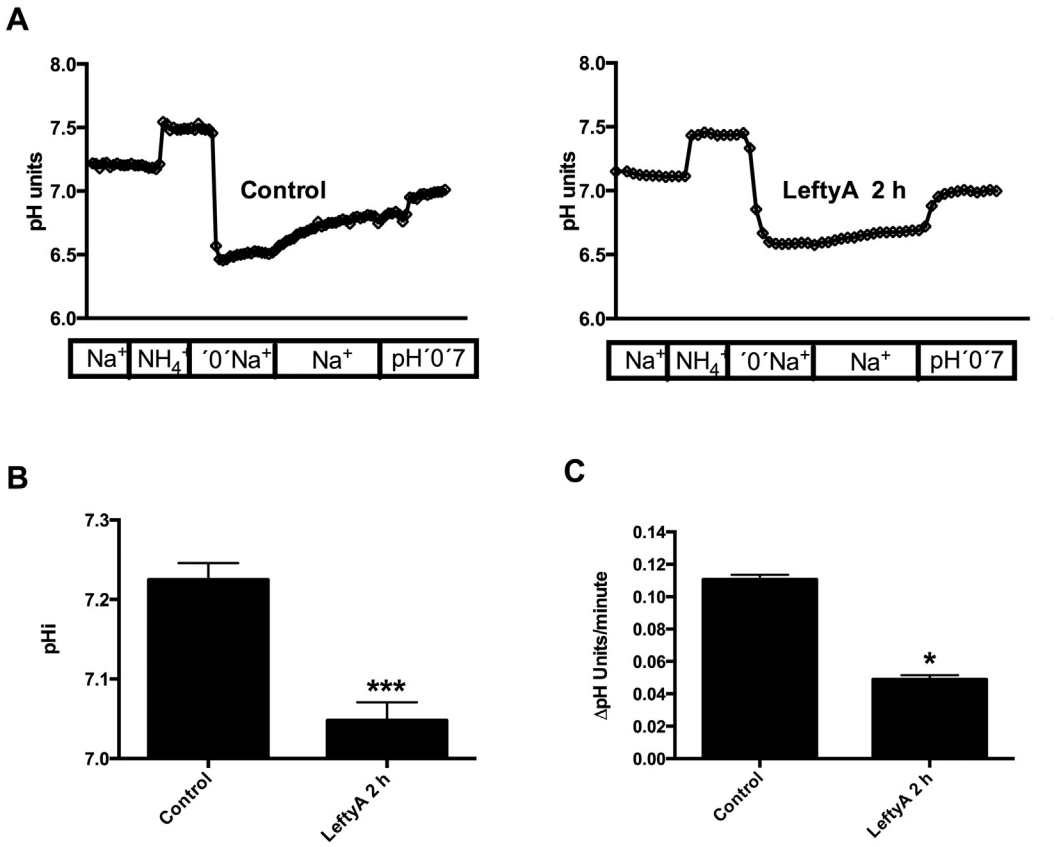

Fig. 3. Effect of LeftyA on pHi and NHE1 activity in Ishikawa cells. **A**. Original tracings of pHi and NHE1 activity, alterations of cytosolic pH (pH_i) in Ishikawa cells prior to, during and following an ammonium pulse. To load the cells with H^+, 20 mM NH_4Cl was added and Na^+ removed (replaced by NMDG) in a first step (see bars below each original tracing), NH_4Cl removed in a second step, Na^+ added in a third step and nigericin (pH_o 7.0) applied in a fourth step to calibrate each individual experiment. **B**. Arithmetic means ± SEM (n = 3 independent experiments) of cytosolic pH prior to the ammonium pulse (pH_i) in Ishikawa cells without or a prior 2 h treatment with LeftyA (8 ng/ml). * indicates statistically significant difference ($P < 0.05$). **C**. Arithmetic means ± SEM (n = 3 independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) in Ishikawa cells with or without 2 h LeftyA (8ng/ml) treatment. * ($P < 0.05$) and *** ($P < 0.0001$) indicates statistically significant difference.

and H^+ if the cells are unable to dispose the H^+ produced by glycolysis. By the same token the inhibition of glycolysis by cytosolic acidification disrupts the major energy source of tumor cells [19,20].

Maintenance of alkaline cytosolic pH is thus decisive for the survival of tumor cells [32–34]. Inhibition of Na^+/H^+ exchanger and cytosolic acidification parallels apoptosis [35], whereby cytosolic acidification fosters the activation of caspases [36]. Accordingly, the presently observed cytosolic acidification may contribute to the known stimulation of apoptosis by LeftyA [13,18].

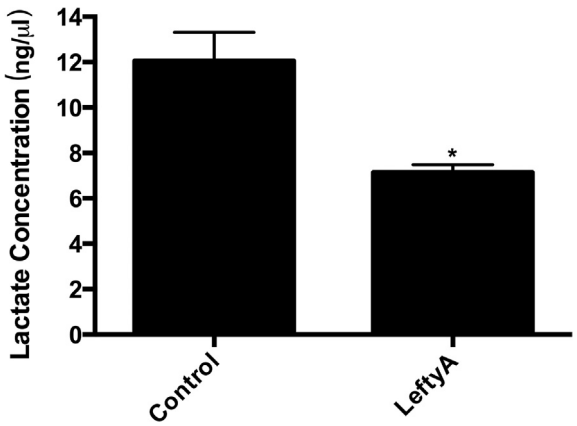

Fig. 4. Effect of Lefty A on lactate concentration in the supernatant of Ishikawa cells. Arithmetic means ± SEM (n = 4) of the lactate concentrations in the supernatant of Ishikawa cells following a 2 h incubation with and without the presence of 8 ng/ml Lefty A. * ($P < 0.05$) indicates statistically significant difference from untreated cells.

Beyond its effect on cytosolic pH, Na^+/H^+ exchanger activity participates in the regulation of cell volume, which involves parallel activity of Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger [37,38]. The carriers mediate the entry of NaCl in exchange for H^+ and HCO_3^-, which are replenished from CO_2 and are thus not osmotically relevant [37,38]. Timely increase of cell volume is a prerequisite for cell proliferation [37,38] and stimulation of cell proliferation is paralleled by a shift of the Na^+/H^+ exchanger cell volume regulatory set point to larger volumes [38].

The present study demonstrates that LeftyA down-regulates expression and function of the Na^+/H^+ exchanger, decreases cytosolic acidification with subsequent impairment of glycolysis. Those effects may well contribute to the anti-proliferative effects of LeftyA.

Conflict of interest

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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