Targeting of GLUT6 (formerly GLUT9) and GLUT8 in rat adipose cells

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The subcellular targeting of the two recently cloned novel mammalian glucose transporters, GLUT6 [previously referred to as GLUT9 [Doege, Bocianski, Joost and Schürmann (2000) *Biochem. J.* **350**, 771–776]] and GLUT8, was analysed by expression of haemagglutinin (HA)-epitope-tagged GLUTs in transiently transfected primary rat adipose cells. Similar to HA-GLUT4, both transporters, HA-GLUT6 and HA-GLUT8, were retained in intracellular compartments in non-stimulated cells. In contrast, mutation of the N-terminal dileucine motifs in both constructs led to constitutive expression of the proteins on the plasma membrane. Likewise, when endocytosis was blocked by co-expression of a dominant-negative mutant of the dynamin

GTPase, wild-type HA-GLUT6 and HA-GLUT8 accumulated on the cell surface. However, in contrast with HA-GLUT4, no translocation of HA-GLUT6 and HA-GLUT8 to the plasma membrane was observed when the cells were stimulated with insulin, phorbol ester or hyperosmolarity. Thus GLUT6 and GLUT8 appear to recycle in a dynamin-dependent manner between internal membranes and the plasma membrane in rat adipose cells, but are unresponsive to stimuli that induce translocation of GLUT4.

Key words: glucose transporter, GLUT4, insulin.

INTRODUCTION

The transport of glucose in most mammalian cells is mediated by a family of facilitative glucose transporters (GLUT1–GLUT4) that display distinct kinetic properties, levels of regulation and tissue distributions [1]. Of particular importance for whole-body glucose homeostasis is the insulin-regulated uptake of glucose into muscle and adipose cells [2]. Both tissues, muscle and fat, contain two closely related glucose-transporter isoforms, GLUT1 and GLUT4, although predominantly the latter. Whereas the ubiquitously expressed GLUT1 is predominantly located in the plasma membrane of most cells, it is partially sequestered within intracellular membrane compartments in insulin-responsive cells. In contrast, GLUT4 is found only in muscle and adipose cells, where it is highly sequestered in an intracellular membrane storage compartment [3]. Insulin stimulation of muscle and adipose cells leads to a rapid and reversible redistribution of GLUT4, and to a lesser extent GLUT1, from intracellular vesicles to the plasma membrane.

Surprisingly, it has been reported previously that certain muscle types in the whole-body GLUT4 knock-out mouse showed a significant insulin-stimulated glucose uptake that cannot be accounted for by an up-regulation of any of the known GLUT isoforms [4,5]. Whereas two novel GLUT-like glucose transporters, GLUT6 [previously referred to as GLUT9 (GenBank®/EMBL Data Bank accession number Y17803); designated SLC2A6 (*Homo sapiens* solute carrier family 2, member 6) by the HUGO Gene Nomenclature Committee] and GLUT8, have been cloned and described recently [6–9], little is known about their subcellular localization, levels of regulation and physiological roles in mammalian cells because the initial studies of these novel GLUTs were performed mainly *in vitro* or in transfected *Xenopus* oocytes. Whereas GLUT8 is expressed mainly in testis, and at lower levels in most other cell types including insulin-sensitive tissues, GLUT6 mRNA was detected only in brain, spleen and leucocytes [6–9]. The aim of the present study was to examine the subcellular targeting of GLUT6 and

GLUT8, and the possible involvement of these two GLUT isoforms in insulin-stimulated glucose transport in a physiologically relevant insulin target cell. Therefore, we have analysed the cell-surface expression of epitope-tagged GLUT6 and GLUT8 in transfected primary rat adipose cells.

MATERIALS AND METHODS

Construction of haemagglutinin (HA)-GLUT6 and HA-GLUT8 mutants

Construction of expression plasmids for HA-GLUT4, HA-GLUT1 and dynamin-K44A (a dominant-negative dynamin mutant) has been described previously [10,11]. Site-directed mutagenesis was performed using the PCR-based QuikChange method (Stratagene, La Jolla, CA, U.S.A.). Cloned *Pfu* DNA polymerase (Stratagene) was used for 16–20 PCR amplification cycles according to the manufacturer's instructions. The cDNAs of GLUT6 and GLUT8 (GenBank® accession numbers Y17803 and Y17802), cloned into pUC18, were used as templates. A unique *NcoI* restriction site was introduced into the cDNAs of GLUT6 and GLUT8 without changing the amino acid sequence of the exofacial loops. The mutagenesis primer (sense strand, mismatches underlined) for GLUT6 was 5'-GGC CTG GAA AGC GCT AGC TGG GGG GAC TTG GCG-3' and that for GLUT9 was 5'-GGC CTG GTG CCC ATC GCT AGC GAG CCT GTG GAT GTC-3'. An oligonucleotide coding for the HA-epitope tag (sense strand: 5'-TAC CCA TAC GAC GTG CCA GAC TAC GCC-3') was then ligated into the *NcoI* sites. The mutagenesis primers for the dileucine (LL)→di-alanine (AA) mutations (sense strand, mismatches underlined) were HA-GLUT6-LL/AA, 5'-GCC ATG CAG GAG CCG GCC GCC GGC GCC GAG GGC CCG-3', and HA-GLUT8-LL/AA, 5'-CAG GAG ACG CAG CCG GCC GCC CGG CCA CCG GAA GCC-3'. Finally, *EcoRI*/*HindIII* fragments of the cDNAs for HA-GLUT6 and HA-GLUT8 were subcloned into the expression vector pcDNA3 (Invitrogen, San Diego, CA, U.S.A.).

Abbreviations used: GLUT, glucose transporter; HA, haemagglutinin.

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All cDNA sequences were verified by automated DNA sequencing.

Cell culture, transfection of rat adipose cells and cell-surface antibody-binding assay

Preparation of isolated rat adipose cells from male rats (CD strain; Charles River Laboratories, Wilmington, MA, U.S.A.), electroporation of rat adipose cells, and the cell-surface antibody-binding assay were performed as described in [11]. Briefly, isolated primary rat adipose cells ($\approx 10^6$ cells/sample) in Dulbecco's modified Eagle's medium were electroporated (three square wave pulses of 12 ms, 500 V/cm) in the presence of 0.25 mg/ml carrier DNA (sheared herring sperm DNA; Roche, Mannheim, Germany) and expression plasmid (1.25–12.5 μ g/ml; 0.4 ml/cuvette). After culturing in Dulbecco's modified Eagle's medium for 24 h at 37 °C in 5% CO₂, the cells were stimulated as described below. KCN was then added to a final concentration of 2 mM and the cells were incubated for 1 h with monoclonal anti-HA antibody (HA.11; Berkeley Antibody Co., Richmond, CA, U.S.A.). After removal of excess antibody, 0.1 μ Ci of ¹²⁵I-labelled sheep anti-mouse antibody (Dupont, Boston, MA, U.S.A.) was added and the cells were incubated for 1 h. Finally, the cells were spun through dinonylphthalate oil to remove unbound labelled antibodies and the cell-surface-associated radioactivity was counted in a gamma counter. Antibody-binding assays were performed in duplicate or triplicate. The values obtained for pcDNA3-transfected cells were subtracted from all other values to correct for non-specific antibody binding.

Western-blot analyses of HA-GLUT4

HA-GLUT-transfected rat adipose cells were harvested after 24 h of protein expression and total cellular membranes were prepared as described previously [10]. The resulting membranes were subjected to SDS/PAGE (12% gel; 25–50 μ g of protein/lane) and transferred on to nitrocellulose filters. The filters were probed with monoclonal anti-HA antibody (1:1000), washed and incubated again with 0.1 μ Ci/ml ¹²⁵I-labelled sheep anti-mouse antibody. Quantification of the blots was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.).

Immunocytochemical detection of HA-GLUT8 overexpressed in COS-7 cells

COS-7 cells were grown on 12 mm glass coverslips, and either transfected with 0.5 μ g of HA-GLUT8 and HA-GLUT8-LL/AA, or co-transfected with 0.4 μ g of HA-GLUT8 and 0.4 μ g of dynamin-K44A with Eugene (Roche). After 48 h, the cells were fixed with formaldehyde, and washed three times in PBS containing 1% BSA. When indicated, the cells were permeabilized in PBS containing 0.5% Triton X-100 for 20 min. Then the cells were washed three times with PBS/1% BSA, blocked in PBS containing 10% normal goat serum (Gibco, Karlsruhe, Germany) for 1 h, and incubated with primary antibody (1:1000) for 1 h at 37 °C. The antibody used was either monoclonal anti-HA antibody or a polyclonal anti-GLUT8 antiserum, raised against the C-terminus (Lys⁴⁶⁷–Arg⁴⁷⁷) of GLUT8 [7]. Thereafter, cells were washed three times with PBS/1% BSA and then incubated for 45 min with FITC-conjugated anti-mouse antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Cells were mounted on coverslips with Fluoromount (Biozol, Eching, Germany) and fluorescence was detected with

a confocal laser scanning microscope (Leica TCS NT; Leica Microsystems, Heidelberg, Germany). FITC was excited at 488 nm using an argon/krypton laser. The FITC fluorescence was selected by a 530 nm band-pass filter. Each optical section was averaged four times.

Statistical analysis

Paired Student's *t* tests were used to compare individual points where appropriate. *P* values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Construction and expression of HA-GLUTs in rat adipose cells

The known members of the GLUT family display similar membrane topology ([1], and see Figure 1). However, whereas GLUT1–GLUT5 contain a large glycosylated extracellular loop between the first and second transmembrane domains, a similar extracellular loop in GLUT6 and GLUT8 is located between transmembrane domains 9 and 10. Moreover, in contrast with the other GLUTs, both GLUT6 and GLUT8 contain N-terminal dileucine (LL) motifs (Figure 1). To allow detection of the two novel GLUTs, we have inserted HA-epitope tags into their large extracellular loops as described in the Materials and methods section. Moreover, to evaluate the targeting potential of the N-terminal dileucine motifs in GLUT6 and GLUT8, we have

Figure 1 Predicted membrane topologies of (A) GLUT4, (B) GLUT6 and GLUT8

(A, B) Localization of the putative glycosylation sites and the epitope tags (HA) is indicated. (C) Predicted cytoplasmic amino acids of the N-termini of human GLUT4, GLUT6 and GLUT8. Dileucine motifs are boxed.

Figure 2 Cell-surface expression of HA-GLUTs in rat adipose cells

Isolated cells were transfected with 0.5 μ g of HA-GLUT plasmid/cuvette and cultured for 20 h at 37 °C. After culturing, the cells were incubated without (basal) or with 67 nM insulin for 30 min, and the cell-surface levels of HA-epitope-tagged glucose transporters were determined using an antibody-binding assay. The cell-surface-associated radioactivity was normalized to the value obtained for insulin-stimulated GLUT4. Results are the means $\pm$ S.D. from duplicate–quadruplicate determinations in 3–10 independent experiments. * P < 0.05 (paired Student's t test) comparing basal and insulin-treated cells. Inset: relative protein-expression levels of HA-GLUTs as determined by Western blotting using anti-HA antibodies (see the Materials and methods section).

constructed mutants where the dileucine motifs were replaced by alanines. Isolated rat adipose cells were transfected with expression plasmids for the HA-GLUTs and cultured for 20 h. Then the cells were harvested and the expression levels of the recombinant HA-GLUT proteins were determined by Western blotting using an anti-HA antibody. As shown in Figure 2, the relative protein expression levels of HA-GLUT4, HA-GLUT6 and HA-GLUT8 were similar. In contrast, the dileucine mutants of both HA-GLUT6 and HA-GLUT8 showed $\approx$ 40–60 % higher expression levels compared with the corresponding wild-type GLUTs.

Cell-surface expression of HA-GLUT constructs

To assess the basal and insulin-stimulated cell-surface expression of the tagged GLUTs, transfected rat adipose cells were harvested after 20 h of cell culture and incubated for 30 min in the absence and presence of insulin. The amount of cell-surface-associated HA-GLUT was then determined in intact cells by an antibody-binding assay against the epitope tag. As illustrated in Figure 2, insulin stimulation of rat adipose cells transfected with HA-GLUT4 induced a $\approx$ 3-fold increase in cell-surface glucose transporters compared with basal values. As described previously, the basal cell-surface expression level of HA-GLUT1 in transfected rat adipose cells was similar to that of HA-GLUT4 in the insulin-stimulated state, and only a small increase in cell-surface HA-GLUT1 was observed upon insulin stimulation [10].

In contrast, despite similar expression levels, the basal cell-surface expression levels of HA-GLUT6 and HA-GLUT8 were only $\approx$ 30 % of that of HA-GLUT4 in the basal state. Moreover, insulin stimulation had no statistically significant effect on the cell-surface expression of either HA-GLUT6 or HA-GLUT8. However, LL $\rightarrow$ AA mutants of both GLUT6 and GLUT8 showed marked increases ($>$ 10-fold) in cell-surface expression compared with the corresponding wild-type constructs (Figure 2). As a result, the cell-surface expression levels of both HA-GLUT6-LL/AA and HA-GLUT8-LL/AA exceeded that of HA-GLUT4 in the insulin-stimulated state. Again, as observed for the corresponding wild-type HA-GLUTs, insulin stimulation of the cells had no effect on the cell-surface expression of both LL $\rightarrow$ AA mutants.

Co-expression of HA-GLUTs and dominant-negative dynamin GTPase

In order to test whether GLUT6 and GLUT8 undergo a constitutive recycling between intracellular compartments and the plasma membrane, we analysed cell-surface HA-GLUTs under conditions where endocytosis is inhibited. Dynamin, a 100 kDa GTPase, has been demonstrated to be an essential component of vesicle formation in both clathrin- and caveolin-mediated endocytosis [12]. Most importantly, co-expression of a dominant-negative dynamin mutant, dynamin-K44A, has been

Figure 3 Co-expression of HA-GLUTs with dynamin-K44A

Isolated cells were co-transfected with 0.5 μ g of HA-GLUT plasmid/cuvette and 0 (—) or 4.5 (+) μ g of dynamin-K44A plasmid/cuvette and cultured for 20 h at 37 °C. After culturing, the cells were incubated without (basal) or with 67 nM insulin for 30 min, and the cell-surface levels of HA-epitope-tagged GLUTs were determined using an antibody-binding assay. The cell-surface-associated radioactivity was normalized to the value obtained for insulin-stimulated GLUT4. Results are the means $\pm$ S.D. from duplicate or triplicate determinations in 2–3 independent experiments. * $P < 0.05$ (paired Student's *t* test) comparing basal and dynamin-K44A-co-transfected cells. Inset: relative protein-expression levels of HA-GLUTs in the absence and presence of dynamin-K44A (K44A) as determined by Western blotting using anti-HA antibodies (see the Materials and methods section).

proven to be a valuable tool for inhibiting general endocytosis of a variety of membrane proteins, including GLUT4 [11–14]. As illustrated in Figure 3, co-expression of dynamin-K44A leads to an increase in cell-surface levels of HA-GLUT4 in both the basal and insulin-stimulated states. As described previously, in the presence of the mutant dynamin the amount of HA-GLUT4 on the plasma membrane exceeded that observed in insulin-stimulated cells in the absence of dynamin-K44A by 20–30 %, even in the basal state, indicating that most of the intracellular HA-GLUT4 appeared to be present on the cell surface, and that insulin had no further effect [11]. Likewise, co-expression of mutant dynamin resulted in a 5-fold increase in cell-surface HA-GLUT8 compared with the control (Figure 3). A similar, albeit smaller, effect (2.5-fold) on the cell-surface expression was observed in cells co-expressing dynamin mutant and HA-GLUT6 (Figure 3). According to Western-blot analyses, the protein-expression levels of HA-GLUT6 and HA-GLUT8 were only slightly reduced in cells co-expressing dynamin-K44A (Figure 3). Interestingly, in cells co-transfected with dynamin-K44A, the levels of HA-GLUT6 and HA-GLUT8 in the plasma membrane did not reach the levels observed in cells expressing

Figure 4 Cell-surface expression of HA-GLUT4, HA-GLUT6 and HA-GLUT8

Cell-surface expression of HA-GLUT4 (empty bars), HA-GLUT8 (hatched bars) and HA-GLUT6 (solid bars) was measured after stimulation of transfected rat adipose cells for 30 min with vehicle (basal), insulin (67 nM), sorbitol (300 mM) or PMA (1 μ M). The cell-surface-associated radioactivity was normalized to the value obtained for insulin-stimulated GLUT4. Results represent means $\pm$ S.D. of cell-surface expression levels obtained from duplicate–quadruplicate determinations in a representative experiment.

the corresponding dileucine mutants alone. In contrast with wild-type HA-GLUT6 and HA-GLUT8, co-expression of mutant dynamin showed no effects on the cell-surface expression of the corresponding dileucine mutants (results not shown).

Stimulation of HA-GLUT translocation in rat adipose cells

Since the subcellular localization of HA-GLUT6 and HA-GLUT8 in rat adipose cells was not affected by insulin, we investigated other potential stimuli that could lead to translocation of the GLUTs to the plasma membrane. In addition to insulin, several other stimuli, such as PMA and hyperosmolarity, have been shown to induce the translocation of GLUT4 to the plasma membrane in adipose cells [15,16]. As shown in Figure 4, incubation of adipose cells in 300 mM sorbitol for 30 min resulted in a significant translocation of HA-GLUT4 to the plasma membrane, almost equal to the effect of insulin. Likewise, stimulation of the cells with PMA increased the cell-surface expression of HA-GLUT4, but to a lesser extent compared with the osmotic shock. However, neither hyperosmolarity nor stimulation with PMA increased cell-surface expression of HA-GLUT6 or HA-GLUT8 (Figure 4).

Subcellular distribution of HA-GLUT8 in COS-7 cells

To test whether the intracellular retention of GLUT8 is due to an intrinsic structural feature of the transporter and not a peculiar result of our assay system, we analysed HA-GLUT8 targeting in transfected COS-7 cells that, unlike adipose cells, lack endogenous GLUT8. The subcellular distribution of the HA-GLUT8 constructs was analysed by confocal microscopy of non-permeabilized and permeabilized cells stained with a monoclonal antibody against the extracellular HA-epitope tag, and permeabilized cells stained with an antibody against the C-terminus of GLUT8 ([7] and see the Materials and methods section). The results are shown in Figure 5. Permeabilized cells expressing wild-type HA-GLUT8 showed a punctuate staining pattern with both antibodies where HA-GLUT8 appeared to be distributed in

Figure 5 Subcellular distribution of GLUT8 expressed in COS-7 cells

Cells were grown on coverslips and either transfected with HA-GLUT8 or HA-GLUT8-LL/AA alone, or co-transfected with HA-GLUT8 and dominant-negative dynamin-K44A. After transfection (48 h), non-permeabilized and permeabilized cells were stained for either the HA-epitope tag or GLUT8 and analysed by confocal microscopy as described in the Materials and methods section. Squares indicate regions of the plasma membrane that are magnified in the right-hand column. The location of the plasma membrane is indicated by arrows. Scale bars, 10 μ m.

intracellular membranes. However, non-permeabilized cells expressing wild-type HA-GLUT8 showed no HA staining (Figure 5). In contrast, cells either expressing HA-GLUT8-LL/AA or co-expressing wild-type HA-GLUT8 and dominant-negative dynamin showed strong cell-surface HA staining under both non-permeabilizing and permeabilizing conditions (Figure 5). Likewise, permeabilized cells stained for GLUT8 showed an increased staining of the plasma membrane region when expressing HA-GLUT8-LL/AA or co-expressing wild-type HA-GLUT8 and dominant-negative dynamin.

DISCUSSION

To address the question of whether the two recently cloned novel members of the mammalian GLUT family, GLUT6 and GLUT8, might be involved in glucose transport in insulin-responsive cells, we have analysed their targeting potential in primary rat adipose cells. Both GLUT8 mRNA and protein are present at low levels in adult muscle and fat tissues, whereas GLUT6 is not expressed in insulin-sensitive tissues [6–9]. The transporters were modified

to contain HA-epitope tags within their large extracellular loops and transfected into freshly prepared adipose cells. The cell-surface expression of the transporters was then analysed by an antibody-binding assay in the intact cells [11]. Because the observed protein-expression levels of the wild-type HA-GLUTs were comparable, the cell-surface binding values obtained actually represent relative subcellular distributions between the plasma membrane and intracellular compartments. Thus, similar to HA-GLUT4, both HA-GLUT6 and HA-GLUT8 are retained in intracellular compartments in non-stimulated cells. However, in contrast with HA-GLUT4, no significant translocation of HA-GLUT6 and HA-GLUT8 to the plasma membrane is observed when the cells are stimulated with insulin. Therefore, our data suggest that in rat adipose cells the two novel GLUTs do not contribute significantly to insulin-stimulated glucose uptake.

Two recent studies have analysed the cell-surface expression of GLUT8 by measuring glucose uptake in transfected *Xenopus* oocytes, which lack endogenous GLUT8 [6,8]. In one study, glucose-transport activity was observed, but only after mutation

of the N-terminal dileucine motif [6]. The other study reported activity without mutating the dileucine motif, but the values were low and the mutated construct was not tested [8]. However, it has been demonstrated previously that GLUT4 and even GLUT1, the latter being expressed mainly on the plasma membrane in many cell types, are strongly retained in intracellular compartments when expressed in *Xenopus* oocytes [17]. Notably, the removal of the C-terminal dileucine motif in GLUT4 that has been implicated as a targeting motif in mammalian cells [18] did not change the subcellular distribution of GLUT4 in the oocytes [17]. Thus the targeting of GLUTs in mammalian and non-mammalian expression systems appears to be different. To assess the role of the N-terminal dileucine motifs in GLUT6 and GLUT8 in insulin target cells, we have transfected rat adipose cells with the corresponding LL → AA mutants and analysed their cell-surface expression. As a result of the mutation, a striking increase in cell-surface HA-GLUT6 and HA-GLUT8 is observed for both mutant GLUTs. Since the cell-surface-associated HA-immunoreactivity of the dileucine mutants exceeds that of HA-GLUT4 in the insulin-stimulated state, it is reasonable to assume that most if not all of the mutant transporters are located in the plasma membrane.

Dileucine motifs have been shown to be involved in internalization and lysosomal targeting of a number of proteins [19]. Since the elevated protein-expression levels (< 2-fold) of the dileucine mutants of both HA-GLUT6 and HA-GLUT8 did not explain the increase (> 10-fold) in cell-surface-associated HA-GLUTs, we concluded that suppression of these motifs results in a decreased rate of internalization, thus trapping the mutant GLUTs in the plasma membrane. Therefore, we have analysed the cell-surface expression of the wild-type GLUTs in rat adipose cells where endocytosis has been blocked by overexpression of a mutant dynamin GTPase [11]. The observed increase in cell-surface expression of HA-GLUT6 and HA-GLUT8 in cells co-transfected with dynamin-K44A demonstrates just such an accumulation of both transporters in the plasma membrane. These data are fully consistent with a recycling of GLUT6 and GLUT8 between the plasma membrane and intracellular compartments. The elevated protein expression levels observed for both dileucine mutants might result from impaired lysosomal targeting and/or a reduced rate of internalization of the mutant transporters that would protect them from entering the degradative pathway.

Consequently, we have attempted to identify possible stimuli that lead to an acute translocation of GLUT6 and GLUT8 to the plasma membrane. Several agents and stimuli have been described to affect the cell-surface expression of GLUT4 in adipose cells independently of the known insulin-receptor signalling pathways, such as phorbol esters and hyperosmolarity [15,16]. However, whereas PMA and osmotic shock led to a translocation of GLUT4 to the plasma membrane, neither of these stimuli increased the cell-surface expression of either HA-GLUT6 or HA-GLUT8. Thus whereas GLUT6 and GLUT8 appear to recycle slowly between internal membranes and the plasma membrane, a physiological stimulus that leads to the regulated translocation of these glucose transporters remains to be identified. Although the physiological roles of GLUT6 and GLUT8 remain uncertain, it has been reported recently that, in blastocysts that do not contain GLUT4, GLUT8 translocates to the plasma membrane in response to insulin [8]. However, our data indicate clearly that in insulin-responsive adipose cells the expression of GLUT6 and GLUT8 does not explain insulin-regulated glucose

transport in the absence of GLUT4. Thus it remains to be shown that cell-specific factors are required for insulin-dependent translocation of GLUT8 in other cell types.

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