

## FIP2 and Rip11 specify Rab11a-mediated cellular distribution of GLUT4 and FAT/CD36 in H9c2-hIR cells

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### Abstract

Rab11a has been shown to be involved in different vesicle trafficking processes. To further define the functional role of Rab11a in vesicle movement we knocked down gene expression of Rab11a and two of its effectors, Rip11 and FIP2, in H9c2-hIR cells and measured the cell surface abundance of GLUT4myc and FAT/CD36. We observed that by knocking down Rab11a, both GLUT4myc and FAT/CD36 abundance at the plasma membrane were substantially increased. In the case of GLUT4myc, the in vitro knockdown of FIP2 also increased the cell surface abundance of GLUT4myc. Knockdown of both FIP2 and Rip11 increase the abundance of FAT/CD36 at the plasma membrane. Stimulated translocation of GLUT4myc and FAT/CD36 is not altered after gene knockdown of Rab11a. These data therefore show that (i) Rab11a regulates cell surface abundance of both GLUT4 and FAT/CD36 and that (ii) both Rab11a-dependent processes are differently regulated by Rab11a effector proteins.

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Among the large number of known Rab proteins, Rab4 and Rab11a have been found to co-localise with GLUT4 in adipose tissue and muscle [1,2]. In contrast to Rab4, the presence of Rab11a was found to increase in GLUT4-containing vesicles after insulin stimulation [2,3]. In this context it was also observed, that localisation of Rab11a after insulin stimulus shifted from microsomal fractions to the plasma membrane. This shift in localisation of Rab11a is coupled to a transient activation of the GTPase, as recently shown by us [4]. In case of GLUT4, the functional role of Rab11a could not be clearly defined so far [5].

A second distinct vesicle population in which Rab11a is found are the FAT/CD36-containing vesicles [6]. FAT/CD36 is the major cardiac fatty acid transporter and has been shown to translocate from intracellular stores, presumably endosomes, to the plasma membrane upon various

physiological stimuli [7]. In contrast to GLUT4-containing vesicles, those vesicles show no increasing amount of associated Rab11a after insulin stimulation. Different observations have been made regarding the insulin sensitivity of FAT/CD36-containing vesicles and their movement to the plasma membrane [6,8]. A similarity of GLUT4-containing and FAT/CD36-containing vesicles is their translocation to the plasma membrane upon electrically induced contraction in cardiac myocytes involving activation of AMP-kinase [9,10].

Given that the function and intracellular localisation of Rab-GTPases is modulated by specific effector proteins, differential regulation of Rab11a in distinct processes can be mediated by proteins of the family of Rab11-interacting proteins (FIPs) [11]. They all share a highly conserved, 20-amino acid motif at the C-terminus of the protein, known as the Rab11-binding domain (RBD) [12]. According to sequence homologies the Rab11a effector proteins are divided into three classes [11]. FIP2, a member of the class I FIPs, regulates recycling of the transferrin receptor

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[13] and functions as an adapter protein for the direct interaction of Rab11a with the motor protein myosin-Vb [14]. A second interesting member of the class I FIPs is Rip11 which also co-localises with Rab11a in endosomal membranes [15].

To investigate the functional role of Rab11a and its effector proteins Rip11 and FIP2 in GLUT4 and FAT/CD36 translocation we established a small interfering RNA (siRNA) knockdown approach in insulin sensitive H9c2-hIR myoblasts. This cell line has been shown to be a good model to study GLUT4 translocation [5,16]. Moreover, glucose uptake directly correlates with the cell surface abundance of GLUT4 in those myoblasts. Subsequently, we have therefore studied the cell surface abundance of GLUT4myc and FAT/CD36 in this cell system. As a stimulus of AMPK-mediated FAT/CD36 translocation hyperosmotic shock induced by sorbitol was used. We observed that by knocking down Rab11a GLUT4 and FAT/CD36 abundance at the plasma membrane is increased already in the basal state. In contrast, stimulated translocation of the metabolite transporters was not altered by knocking down Rab11a, suggesting that Rab11a has no effect on the stimulated exocytosis of neither GLUT4 nor FAT/CD36, but regulates the retention of both metabolite transporters at the plasma membrane. Gene knockdown of FIP2 and Rip11 increased cell surface abundance of GLUT4 and FAT/CD36 unequally, suggesting a differential regulation of Rab11a in both processes by Rab11-interacting proteins.

## Materials and methods

**Materials.** The pcDNA3.1 plasmid coding for FAT/CD36 was gifted by M.M. van Oort (Utrecht University, The Netherlands). FAT/CD36 was detected in Western blot analyses using the MO25 antibody kindly provided by Dr. N.N. Tandon (Otsuka Maryland Research Institute, Rockville, USA). The GLUT4myc cDNA was a gift from Dr. A. Klip (University of Toronto, Canada) and ligated into the NotI restriction site of pCMV vector as described earlier [5]. Published siRNA sequences against rat Rab11a (UGUCAGACAGACGUGAAAAdTdT), Rip11 (GCGGACUGGAGAGGCUCAGAAdTdT) and FIP2 (GUCUAGCA CUGUAGGCCAdTdT) were purchased from QIAGEN (Hilden, Germany). 5-aminoimidazole-4-carboxamide-1- $\beta$ -riboside (AICAR) was from Calbiochem (Darmstadt, Germany), insulin, D-sorbitol and 2,4-dinitrophenol were from Sigma-Aldrich (Seelze, Germany). Primary antibodies against Rip11 and FIP2 were kindly provided by R. Prekeris (University of Colorado Health Sciences Center, USA). Antibodies against Rab11a were from BD Biosciences (Heidelberg, Germany), antibodies against GLUT4 (C-20) and actin (C-2) were from Santa Cruz (Heidelberg, Germany), antibodies against c-myc (9E10) were produced by Calbiochem (Darmstadt, Germany) and antibodies against murine CD36 were from Cascade Biosciences (Winchester, USA). Primary antibodies against phospho-AMPK $\alpha$  (Thr172) were from Cell Signaling (Danvers, USA).

**Cell culture of H9c2-hIR and transfection.** H9c2-hIR (clone E2) cells were generated as previously described [5]. The cells were cultured in DMEM with 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, non essential amino acids and 0.6 mg/ml G418. Twenty-four hours before plasmid transfection cells were seeded with  $35 \times 10^3$  cells per well of a 12-well plate. Transfection of siRNA was done 6 h after seeding with 20 nM siRNA and 2  $\mu$ l Lipofectamin 2000 per well in antibiotic free culture medium. Transfection of plasmid DNA was carried out 24 h after seeding with 0.5  $\mu$ g DNA and 1.5  $\mu$ l FuGene6 per well in culture medium. Sixteen

hours after plasmid transfection cells were washed and medium was changed. Forty-eight hours after plasmid transfection translocation assay or immunoblotting following lysis was performed.

**GLUT4myc translocation assay.** The assay of GLUT4 translocation is based on the method described by Kanai et al. [17]. After 48 h of GLUT4myc expression, cells were incubated with or without 1  $\mu$ M insulin in incubation buffer for 30 min at 37 °C. Then, the cells were fixed with 2% paraformaldehyde for 30 min at room temperature and blocked in PBS containing 5% fatty free milk for 1 h. The fixed cells were incubated with antibodies against c-Myc in a dilution of 2  $\mu$ g/ml in PBS containing 5% fatty free milk for 2 h followed by incubation with secondary  $^{125}$ Iodine labelled antibodies against mouse-IgG (0.1  $\mu$ Ci per well) for 1 h. After washing with PBS containing 5% fatty free milk, PBS and PBS containing 0.05% Tween 20, the cells were solubilised with 1% SDS and the radioactivity was measured using a  $\gamma$ -counter (1282 Compugamma CS, Wallac Oy, Turku, Finland).

**FAT/CD36 translocation assay.** The assay of FAT/CD36 translocation is adapted to the assay of GLUT4myc translocation with minor modifications. Translocation of FAT/CD36 was induced with 0.6 M sorbitol instead of insulin. The blocking solution contained 1% BSA instead of 5% fatty free milk and the primary antibodies were against CD36 in a dilution of 2  $\mu$ g/ml in blocking solution.

**Lysis and immunoblotting.** For lysis of the cells, buffer containing 50 mM Tris-HCl (pH 7.4), 0.5% (v/v) Triton X-100, 2 mM MgCl $_2$ ·6H $_2$ O, 150 mM NaCl, 2% (v/v) Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany), 1 mM Na $_3$ VO $_4$  and 1 mM NaF was added into the wells and incubated for 5 min on ice. After preparation, lysates were boiled in Laemmli buffer and separated by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After blocking in TBS containing 0.05% Tween 20 and 5% fatty free dry milk, membranes were incubated with the appropriate primary antibody at 4 °C overnight. After extensive washing, membranes were incubated with corresponding horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualised by the enhanced chemiluminescence method on a Lumi-Imager work station (Roche Diagnostics, Mannheim, Germany).

**Statistics.** All data are presented as means  $\pm$  SEM. Statistical analysis was performed by using Student's *t*-test or ANOVA and statistical analysis software Prism 4 (GraphPad Software, Inc.). A *p* value of less than 0.05 was considered to be statistical significant, a *p* value less than 0.01 to be very significant. Corresponding significance levels are indicated in the figures.

## Results

### Overexpression and gene knockdown

To measure GLUT4 translocation to the plasma membrane in H9c2-hIR cells, it is necessary to overexpress the myc-tagged GLUT4 protein. When GLUT4myc is integrated into the plasma membrane, this myc-epitope is located extracellularly and can be bound by an appropriate antibody. FAT/CD36 can be detected in its endogenous conformation, still it needs to be overexpressed in H9c2-hIR cells. To make sure that overexpression of GLUT4myc and FAT/CD36 does not interfere with gene knockdown of Rab11a, Rip11 and FIP2, Western blot analyses were carried out before measuring translocation.

Both single transfection with specific siRNA and cotransfection with pCMV-GLUT4myc lead to a gene knockdown of Rab11a by 76.0 ( $\pm$  4.0)% and 79.0 ( $\pm$  3.8)%, respectively (Fig. 1A). Also Rip11 gene expression can independently of GLUT4myc expression be

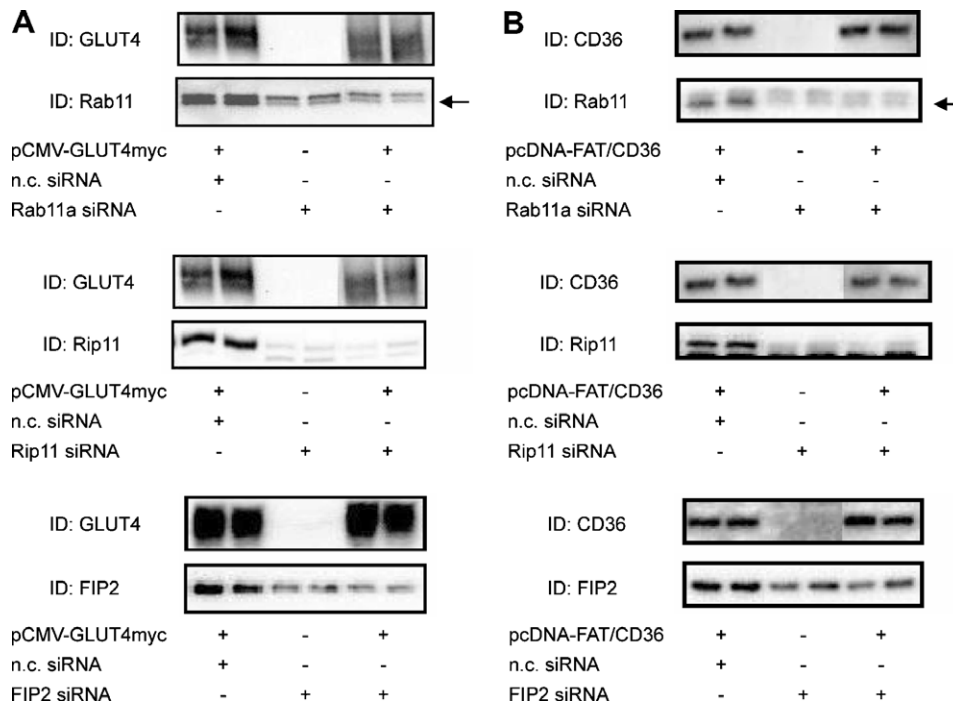


Fig. 1. RNAi induced gene knockdown and overexpression. Six hours after seeding H9c2-hIR cells were transiently transfected with 20 nM non-coding siRNA (n.c. siRNA), Rab11a siRNA, Rip11 siRNA and FIP2 siRNA, respectively. Twenty-four hours after seeding cells were transfected with pCMV-GLUT4myc (A) or pcDNA-FAT/CD36 (B), respectively. Forty-eight hours after plasmid transfection cells were lysed. Lysates were separated by SDS-PAGE and immunoblotted for Rab11a, Rip11, FIP2, GLUT4 and FAT/CD36 and normalised for tubulin (not shown). Representative blots each done in duplicate are shown. Western blots were quantified on a Lumi-Imager work station, as detailed in Materials and methods.

reduced by  $95.0 (\pm 1.0)\%$  and  $96.0 (\pm 1.0)\%$ , respectively. Similar results were observed for knockdown of FIP2.

The cotransfection with pcDNA-FAT/CD36 also does not interfere with gene repression of Rab11a, Rip11 and FIP2. The expression of Rab11a is reduced by  $81.0 (\pm 4.6)\%$  and  $87.1 (\pm 4.6)\%$ , respectively. Rip11 can be reduced by  $76.0 (\pm 8.7)\%$  and  $79.8 (\pm 8.0)\%$ , respectively, and FIP2 expression is reduced by  $51.0 (\pm 7.0)\%$  and  $56.0 (\pm 7.0)\%$ , respectively (Fig. 1B).

#### Activation of AMP-kinase in H9c2-hIR cells

While it has already been shown that insulin induces GLUT4myc translocation in H9c2-hIR cells [5], it was not possible to induce FAT/CD36 translocation in this cell system with the same stimulus (data not shown). Because of that, different compounds were tested to activate AMP-kinase in H9c2-hIR cells. To do so, 24 h after seeding cells were treated for 30 min with 2 mM AICAR, 0.6 M D-sorbitol and 0.1 mM 2,4-dinitrophenol, respectively. Insulin was used as a negative control.

Surprisingly, neither AICAR nor 2,4-dinitrophenol treatment was able to activate AMP-kinase in H9c2-hIR cells significantly. However, D-sorbitol treatment leads to an increase in AMP-kinase phosphorylation at Thr172 by  $2.2 (\pm 0.26)$  fold ( $p < 0.05$  SEM,  $n = 3$ ) (Fig. 2). The lack of an effect of AICAR on AMPK-phosphorylation might

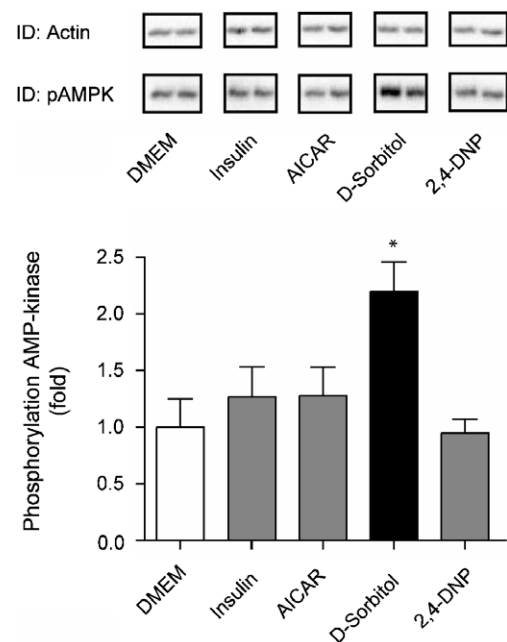


Fig. 2. Activation of AMP-kinase. Almost confluent H9c2-hIR cells were treated for 30 min with 2 mM AICAR, 0.6 M D-sorbitol and 0.1 mM 2,4-dinitrophenol (2,4-DNP), respectively. Insulin was used as a negative control. Afterwards cells were lysed, lysates were separated by SDS-PAGE and immunoblotted for phospho-AMP-kinase and actin. Representative blots each done in duplicate are shown. Western blots were analysed on a Lumi-Imager work station, as detailed in Materials and methods. Data are mean values  $\pm$  SEM ( $n = 3$ ). \* $p < 0.05$ .

show a putative absence of adenosine kinase, which converts AICAR to AICAR-monophosphate (ZMP), which is necessary for AMPK-activation [18]. With respect to 2,4-dinitrophenol, we cannot explain why this uncoupler did not result in AMPK activation. Hence, for activation of AMPK in H9c2-hIR cells we used hyperosmotic shock induced by sorbitol, which is already known for a long time to increase AMPK activation and glucose uptake [19,20].

#### *Rab11a regulates GLUT4 cell surface abundance in concert with FIP2*

Cotransfection of GLUT4myc and non coding siRNA represents the control situation for results presented in Fig. 3. Treatment of H9c2-hIR cells with insulin leads to an increase of GLUT4myc translocation by  $54 (\pm 6)\%$  (Fig. 3A). Knockdown of Rab11a increases GLUT4 cell surface abundance by  $59 (\pm 10)\%$  compared to the basal control. In contrast to Rab11a, Rip11 gene knockdown

has no significant effect on GLUT4myc sequestration. On the other hand, even stronger effects than in the case of Rab11a knockdown are seen when repressing the FIP2 gene expression. Here the GLUT4myc cell surface localisation is increased by  $136 (\pm 6)\%$  (Fig. 3A).

While the total amount of GLUT4myc mobilised by insulin is increased in the cases of Rab11a and FIP2 gene repression (Fig. 3A and B), the relative increase of GLUT4myc translocation induced by insulin compared to the corresponding basal values remains unaltered (Fig. 3C).

#### *FAT/CD36 cell surface abundance is regulated by Rab11a, FIP2 and Rip11*

While neither insulin nor AICAR did change the translocation of FAT/CD36 in H9c2-hIR cells (data not shown), sorbitol led to an increase of  $38 (\pm 13)\%$  in the control situation (Fig. 4A). Knocking down gene expres-

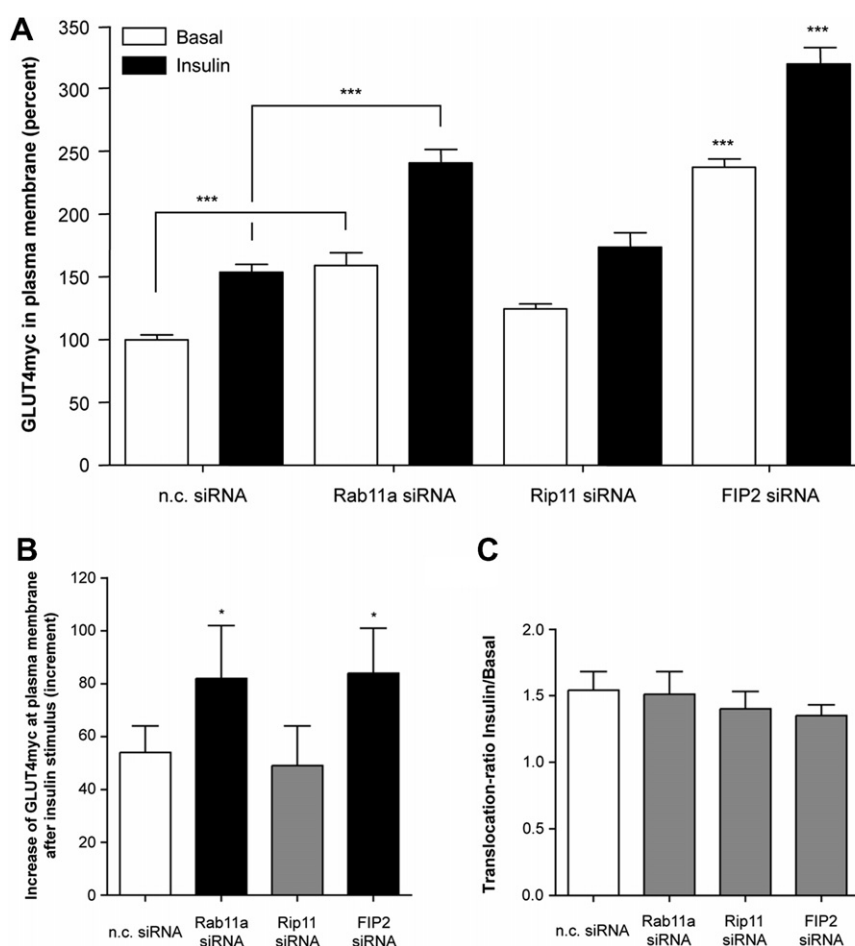


Fig. 3. Cell surface abundance of GLUT4myc. Six hours after seeding H9c2-hIR cells were transiently transfected with 20 nM non-coding siRNA (n.c. siRNA), Rab11a siRNA, Rip11 siRNA and FIP2 siRNA, respectively. Twenty-four hours after seeding cells were transfected with pCMV-GLUT4myc. Forty-eight hours after plasmid transfection cells were incubated with or without insulin and abundance of GLUT4myc at the plasma membrane was measured as detailed in the methods section. (A) Abundance of GLUT4myc at plasma membrane, basal value of n.c. siRNA set to 100%. (B) Total increase of GLUT4myc at plasma membrane after insulin stimulation in dependency of gene knockdown. Values represent increments calculated from (A). (C) Relative activation of GLUT4myc translocation by insulin related to corresponding basal values. Data are mean values  $\pm$  SEM ( $n = 3$ ). \*\*\* $p < 0.001$ , \* $p < 0.05$ .

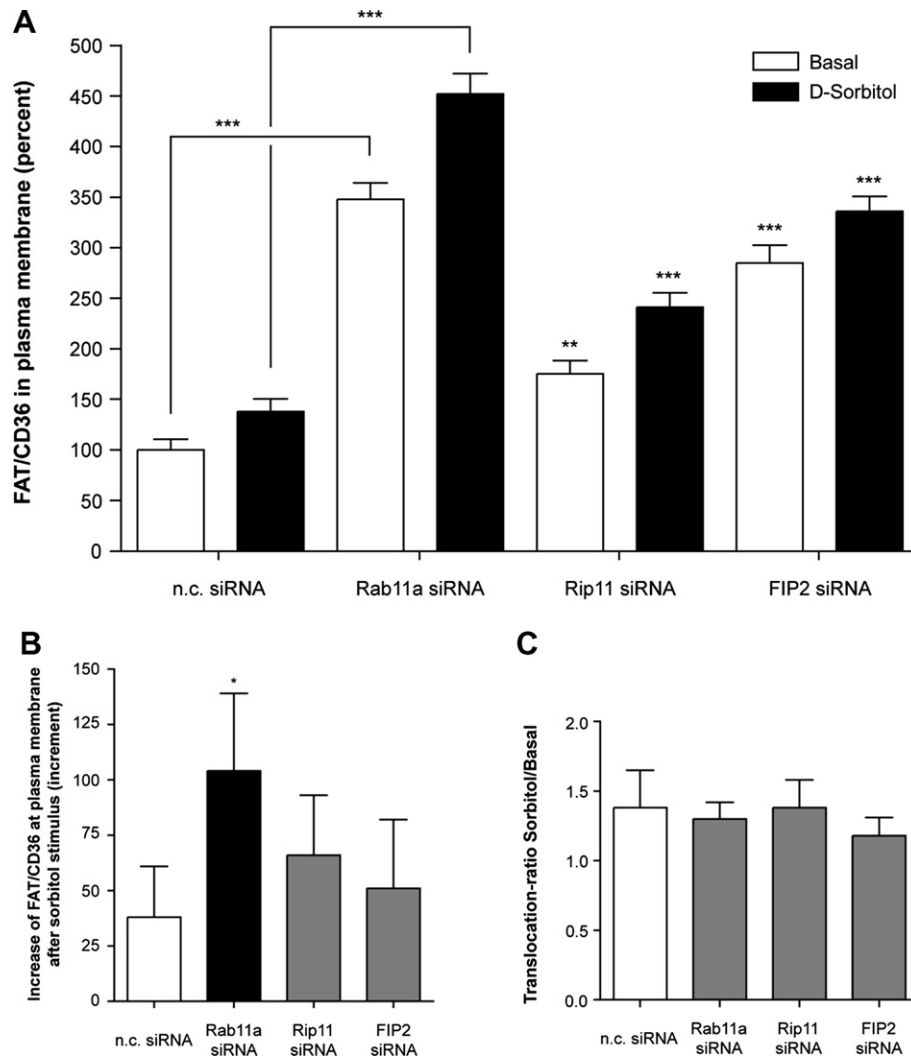


Fig. 4. Cell surface abundance of FAT/CD36. Six hours after seeding H9c2-hIR cells were transiently transfected with 20 nM non-coding siRNA (n.c. siRNA), Rab11a siRNA, Rip11 siRNA and FIP2 siRNA, respectively. Twenty-four hours after seeding cells were transfected with pcDNA-FAT/CD36. Forty-eight hours after plasmid transfection cells were incubated with or without sorbitol and abundance of FAT/CD36 at the plasma membrane was measured as detailed in the methods section. (A) Abundance of FAT/CD36 at plasma membrane, basal value of n.c. siRNA set to 100%. (B) Total increase of FAT/CD36 at plasma membrane after sorbitol stimulation in dependency of gene knockdown. Values represent increments calculated from (A). (C) Relative activation of FAT/CD36 translocation by sorbitol related to corresponding basal values. Data are mean values  $\pm$  SEM ( $n = 3$ ). \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ .

sion of Rab11a increased the basal FAT/CD36 cell surface abundance by 248 ( $\pm 16$ )%. In contrast to GLUT4myc, the knockdown of Rip11 increases the redistribution of FAT/CD36 significantly by 75 ( $\pm 13$ )%. Gene repression of FIP2 increases the basal FAT/CD36 cell surface localisation by 185 ( $\pm 17$ )%. In contrast to their influence on GLUT4myc transport, those effects are weaker than the effects of the Rab11a gene knockdown.

Under conditions of Rab11a gene knockdown the absolute amount of FAT/CD36 activated by sorbitol to translocate to the plasma membrane is increased while Rip11 and FIP2 knockdown only show a tendency to increase the induced translocation (Fig. 4B). The relative sorbitol induced translocation in relation to the basal values remains unaffected, showing like in case of GLUT4myc only an influence of Rab11a on the re-entry of FAT/CD36 (Fig. 4C).

## Discussion

By knocking down gene expression of Rab11a and its effector proteins FIP2 and Rip11 in our present study, we could modulate the GLUT4myc and FAT/CD36 abundance at the cell surface. While the absolute amount of GLUT4myc translocated by insulin is increased in the case of Rab11a knockdown, the insulin stimulated increase of the exocytosis/endocytosis rate of GLUT4 remains equal. Rab11a therefore has no effect on the insulin-stimulated exocytosis of GLUT4 but rather regulates the re-entry of the glucose transporter into the endosomal compartment. This has been postulated in earlier publications [5,21]. In an initial experiment we could confirm that the cell surface abundance of GLUT4 directly correlates with glucose uptake in H9c2 cells (data not shown) [5,16].



The basal state is characterised by a slow exocytosis and more rapid endocytosis of GLUT4 [22,23]. The gene knockdown of Rab11a may decrease the rate of endocytosis in the basal state, so that the net translocation of GLUT4 is increased. The observed activation of Rab11a by GTP loading in response to insulin obviously takes place to attach Rab11a to GLUT4-containing vesicles [4]. This might be needed for enhanced endocytosis and re-sequestration of GLUT4 into its storage compartments after termination of the insulin signal, but is not involved in the actual translocation process.

FIP2 knockdown increases the basal GLUT4 abundance and the insulin stimulated GLUT4 translocation significantly. It is therefore concluded that Rab11a regulates the re-entry of GLUT4 into the cell in association with its effector protein FIP2. Activated Rab11a interacts with the motorprotein myosin-Vb and thereby regulates the recycling of early endosomes along the actin filaments [24]. The interaction of Rab11a with myosin-Vb is enabled by its association to FIP2 [14]. This ternary complex is known to recycle the transferrin receptor and the chemokine receptor CXCR2 [13,25]. Our hypothesis is that after termination of the insulin signal Rab11a binds to FIP2 to initiate internalisation of GLUT4. The complex of Rab11a and FIP2 could lead to association of myosin-Vb to move early endosomes carrying GLUT4 to the endosomal recycling compartment.

In contrast to insulin, sorbitol-treatment resulted in translocation of FAT/CD36 in H9c2-hIR cells. Although overexpression of FAT/CD36 does not increase fatty acid uptake in H9c2 cells [26], it still is a useful cell model to investigate stimulated translocation of FAT/CD36 vesicles into the plasma membrane. In the present study gene knockdown of Rab11a increased the FAT/CD36 abundance at the plasma membrane under basal conditions, and also further enhanced its presence in sorbitol treated cells. Rab11a knockdown influences subcellular FAT/CD36 trafficking very similar to that of GLUT4. The combined findings that Rab11a knockdown increases the redistribution of both GLUT4 and FAT/CD36 to the cell surface, but leaves the induced translocation of both transporters unaltered, strongly suggests that Rab11a has a similar function in internalisation of GLUT4 and FAT/CD36.

Different observations have been made regarding the influence of the proteins Rip11 and FIP2 in Rab11a-mediated internalisation. While Rip11 has no effect on GLUT4 translocation, it shows an influence on FAT/CD36 redistribution. This would suggest a different regulation of Rab11a by a different combination of Rab11a-effector proteins being involved in FAT/CD36 sequestration. While GLUT4 is clearly redistributed by Rab11a in complex with FIP2, the internalisation of FAT/CD36 seems not to be completely mediated by a single Rab11a effector protein. To further elucidate the regulation of FAT/CD36 internalisation by Rab11a, a wider approach containing more members of the Rab11-family of interacting proteins is needed.

In conclusion, the data of the present study show that Rab11a regulates re-entry of both GLUT4 and FAT/

CD36 differently by association of specific Rab11a-interaction proteins. While Rab11a-mediated GLUT4 internalisation might be mediated in concert with FIP2, FAT/CD36 redistribution needs a more complex composition of Rab11a effector proteins which still has to be defined.

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