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SWAP70 undergoes dynamic conformational regulation at the leading edge of migrating cells

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ABSTRACT:

Rearrangements of the actin cytoskeleton are regulated in part by dynamic localised activation and inactivation of Rho family small GTPases. SWAP70 binds to and activates the small GTPase RAC1 as well as binding to filamentous actin and PIP₃. We have developed an encoded biosensor, which uses Forster Resonance Energy Transfer (FRET) to reveal conformational changes in SWAP70 in live cells. SWAP70 adopts a distinct conformation at the plasma membrane, which in migrating glioma cells is enriched at the leading edge but does not always associate with its PIP₃-dependent translocation to the membrane. This supports a role for SWAP70 in positive feedback activation of RAC1 at sites of filamentous actin, PIP₃ and active RAC1.

KEYWORDS: SWAP70, RAC1, FRET, PHOSPHOINOSITIDE.

INTRODUCTION

Rho family small GTPases regulate many aspects of cell adhesion and cytoskeletal organisation [1-3]. Their localised, dynamic and reversible switching between inactive and active states is under complex control with key players being activating Guanine Nucleotide Exchange Factors (GEFs) and inactivating GTPase activating Proteins (GAPs). Canonical GEFs promote release of bound GDP from the GTPase, allowing binding of abundant GTP and activation, whereas GAPs promote GTP hydrolysis, leading to an inactive state [3].

SWAP70 (Switch Associated Protein - 70) is a multi-domain protein containing an N-terminal potentially calcium-binding EF hand domain, a Pleckstrin Homology (PH) domain able to bind selectively to PIP_3 and $PI(3,4)P_2$, a Dbl Homology (DH) domain and a C-terminal actin-binding motif. The PH and DH domain sequences show that SWAP70 is related to a conserved family of GEFs although its activity to catalyse exchange is controversial [4-6]. First identified as part of the B lymphocyte class switching complex [7], SWAP70 is expressed widely and plays roles which influence several distinct cellular processes. These include cell migration, adhesion and invasion [6, 8, 9], phagocytosis [4, 10], endothelial permeability [11] and bone resorption and podosome formation in osteoclasts [12, 13]. The molecular basis of many of these cellular functions seems to be local activation of small GTPases, particularly RAC1. It has been proposed that this is achieved directly by GEF activity of the SWAP70 DH domain, which is evolutionarily conserved across many eukaryotes including plants [14, 15] although the observed strong binding of SWAP70 to RAC1-GTP and its weak binding to the unliganded GTPase support other proposed mechanisms by which SWAP70 promotes RAC1 activation. Additionally, potentially GEF-independent functions including bundling and tethering actin have also been proposed [10, 16].

The localised activation of RAC1 by a large and diverse group of GEFs has been heavily studied particularly in the context of cell migration. Several RAC1 activating GEFs have been shown to bind to the Class I Phosphoinositide 3-Kinase (PI3K) lipid product phosphatidylinositol 3,4,5-trisphosphate (PIP_3) thus mediating the frequently observed PI3K-dependent activation of RAC1 [17, 18]. Furthermore, demonstration of the direct activation of the PI3K catalytic subunit $p110\beta$ by RAC1 has helped provide a stronger mechanistic basis for proposed positive feedback loops involving activated PI3K and RAC1 [19-22].

The localisation of the active and inactive pools of GTPases and the proteins which regulate them is key to their function and experimental methods to reveal these activation events in living cells are challenging but rewarding [23, 24]. Here we describe the construction of a FRET based reporter, which allows the first analysis of the dynamic regulation of SWAP70 in living cells. This reveals local conformational changes in SWAP70, which mirror the activation of RAC1 at the leading edge of migrating cells and provides new insights into its function.

MATERIALS AND METHODS

Plasmid constructs and cloning

The FRET standards: C5V and CTV; and control vector mVenus-C1 were purchased from Addgene. The CFP control vector, mTurquoise-C1, was subcloned from the FRET vector backbone F35 (kindly provided by Dr Carsten Schultz, EMBL [25]). The SWAP70 protein expressed throughout this study is human SWAP70 isoform 1, Consensus Coding Sequence CCDS31426. The FRET biosensor (later termed SWAP70-R) for wild-type SWAP70 was cloned by PCR amplification from a SWAP70 cDNA provided by the I.M.A.G.E. consortium, using primers 5' GACCGGTGGGAGCTTGAAGGAGGAGCTGC 3' and 5' CAACGCGTCTCCGTGGTCTTTTTCTCTTTCCAG 3' and then inserting it into FRET backbone F35 using AgeI and MluI. SWAP70-R230C FRET biosensor was created by site directed mutagenesis of SWAP70-WT FRET biosensor using primers: 5' AAAACTGGACTGAATGTTGGTTTGTTTTAAACCCAA 3' and 5' TTGGGTTTTAAACAAACCAACATTCAGTCCAGTTTT 3'.

Cell culture

All the cells lines used in this study were cultured in DMEM supplemented with 10% FBS (Sigma), 2 mM glutamine (Life Technologies) and non-essential amino acids (Life Technologies) at 37°C in 5% CO₂ 95%air (v/v). For microscopy experiments cells were cultured and maintained at low cell density (20%-30%) in phenol red free media.

Western blotting

For protein expression analysis, cell lysates were prepared by using ice-cold lysis buffer (25mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EGTA, 1 mM EDTA, 5 mM sodium pyrophosphate, 10 mM beta-glycerophosphate, 50 mM sodium fluoride, 0.2 mM PMSF, 1 mM benzamidine, 0.1% betamercaptoethanol) followed by centrifugation at 14,000 rpm for 20 minutes at 4°C. Supernatants were subjected to protein estimation using Bradford's assay. Equal amounts of proteins from each lysate were resolved by SDS-PAGE using pre-cast 4%- 12% Bis-Tris gels (Life Technologies), transferred onto methanol activated PVDF membrane (Perkin Elmer) and blotted for SWAP70 and GAPDH using their respective primary antibodies (α -SWAP70, 1:1000, abcam #89605; and α -GAPDH, 1:10,000, Millipore #MAB374). Bound primary antibodies were detected using HRP-conjugated goat anti-mouse IgG antibody (Invitrogen, Thermo scientific).

Cell transfection and staining

For all microscopy experiments, cells (150,000 cells in case of U87MG or 300,000 cells in case of Swiss-3T3 cells) were seeded onto 25 mm cover slips placed in each well of six well plate. Cells were transfected 24 hours later using Lipofectamine-2000 (Life Technologies) in Opti-MEM (Life Technologies) as per manufacturer's

instructions. 24 hours post-transfection media was changed and 48 hours post-transfection cells were imaged live or fixed. For some experiments Swiss-3T3 cells were serum starved after 48 hours of transfection for a further 24 hours before imaging or fixation. Similarly, for some experiments, U87MG cells were treated with some inhibitors 48 hours post-transfections before imaging or fixation. For each FRET experiment, cells were transfected with plasmid encoding FRET biosensor and also with control vectors: mTurquoise-C1 and mVenus-C1 for SBT calculation purpose; and with FRET standards: C5V and CTV. For fixed cell experiments, cells expressing Venus or SWAP70 (WT or R230C) were permeabilised using 0.2% Triton in PBS for 5 minutes followed by two washes with PBS. The permeabilised cells were then stained for actin using Alexa Fluor 633 Phalloidin (Thermo Fisher Scientific) for 20 minutes at RT followed by two washes with PBS.

Live cell imaging

All the live cell imaging or FRET experiments were performed on Olympus IX-81 wide-field microscope equipped with Olympus CellXcellenceR acquisition software, an ImageEM EM-CCD 512 x 512 camera (Hamamatsu UK) and an Olympus X 60 APON 1.45 NA oil lens with a resulting pixel size of 266 nm. For data acquisition, cells expressing FRET probes, FRET standards or Donor (i.e. mTurquoise) or acceptor (i.e. Venus) were imaged in three different channels: (1) Donor channel: excitation 430 nm and emission 470 nm (i.e. excitation peak and emission peak respectively for mTurquoise and Cerulean), (2) Acceptor channel: ex. 500 nm and em. 530 nm (i.e. excitation peak and emission peak respectively for Venus), and (3) FRET channel: ex. 430 nm and em. 530 nm. The excitation source (150W mercury-xenon MT20-E illumination system) intensity, EM gain, and exposure time were the same for donor excitation and acceptor excitation in all the samples across one experiment.

Fixed cell imaging

Cells were fixed using 4% paraformaldehyde in PBS for 20 minutes at RT, followed by two washes with PBS. All fixed cell imaging was performed on Leica SP5 SMD confocal laser scanning microscope (CLSM) using a 63X 1.4 NA HC PL Apo oil immersion objective lens and a pinhole set to 1 Airy unit. 512 nm and 633 nm lasers were used to excite Venus and Alexa Fluor 633 Phalloidin probes, respectively. Emission was detected using a photomultiplier tube (PMT) set to a gain of 600-800 V.

Image processing and analysis

All image processing and analysis were performed using Fiji (ImageJ). The FRET efficiency images were generated using an ImageJ based plugin PFRET (purchased from KCCI, UVa, Virginia, USA).

RESULTS

Development of a FRET-based SWAP70 conformation reporter

The activity state of SWAP70 and other multi-domain GEF family members is thought to be regulated through localised transient binding to specific regulatory lipids and proteins [1]. For most of these proteins, including SWAP70, protein modifications such as phosphorylation which are stable and correlate with activity have not been identified, making it difficult to analyse the activation state of these proteins. A simple hypothesis seems that the regulation of SWAP70 is associated with significant changes in the conformation of the enzyme upon local binding to partner proteins and lipids. Therefore, we elected to design encoded reporter constructs with the aim of revealing transient localised changes in protein conformation by engineering modified SWAP70 proteins with cyan and yellow fluorescent proteins (CFP and YFP) fused to the N and C-termini respectively. This allows localised changes in protein conformation and the relative position of the two termini to be revealed by changes in the efficiency of FRET occurring between the CFP and YFP, and has been shown to be successful with other proteins [25, 26]. We used a large set of previously described CFP-YFP constructs designed using mTurquoise and Venus fluorescent proteins (hereafter simplified as CFP and YFP respectively) with a range of different linkers [25]. Into this backbone series, we inserted a cDNA encoding one of several reported PIP₃-regulated GEF-family members: SWAP70, DEF6 or TIAM1. 21 different CFP-GEF-YFP expression vectors were constructed, transiently transfected into human U87MG glioma cells and subjected to FRET analysis in unselected live cells. Most of the 21 constructs were rapidly rejected because FRET was either very low or because uniform FRET was observed in every cell analysed. This implies either that systematic localised conformational changes are not occurring in these proteins in the conditions of the experiment or that the linkage to fluorophores fails to couple changes in protein conformation with changes in detectable FRET in these constructs. In contrast, several reporters based on SWAP70 showed non-uniform FRET in a large proportion of cells and were taken forward for further analysis.

In the characterisation of these reporter constructs, positive (maximum) and negative (minimum) CFP-YFP control FRET constructs were used: C5V has a 5 amino acid linker between the fluorescent proteins, showing consistently high FRET whereas CTV has a 229 amino acid linker and reports very low FRET (Figure 1). The ability to detect dynamic changes in FRET was also tested using a previously characterised CFP-DAPK1-YFP control [25] which shows greatly reduced FRET within 3 minutes in cells treated with the calcium ionophore ionomycin (Figure S2). The FRET efficiency observed with CFP-SWAP70-YFP (hereafter called SWAP70-R) was always intermediate between these two controls but in many cells displayed elevated FRET efficiency at the cell periphery. Importantly, these areas of elevated FRET were frequently observed in locations, which showed no evidence of enrichment of the reporter itself, visualised through either direct YFP or CFP fluorescence (Figure

1). The converse observation that regions of the cell enriched in SWAP70-R protein frequently displayed low FRET also argues strongly that the FRET signal is not simply caused by high local concentrations of the fluorophores. Furthermore, this non-uniform FRET pattern was not observed with other constructs capable of FRET in these cells (e.g. C5V or DAPK1 controls, Figures 1C, S2, S3).

SWAP70 regulates lamellipodia

SWAP70 activity has been shown to promote the formation of cellular lamellipodia and membrane ruffles [6, 16]. The full length protein was over-expressed in the SWAP70-R format, in U87MG glioma cells, which were selected for their low expression of the endogenous protein among other glioblastoma cell lines [27] (Figure S1). SWAP70-R was seen to be enriched on the peripheral cell membrane of many but not all cells and this over-expression led to the formation of prominent filopodia and lamellipodia on a substantial minority of cells (Figure 2). Notably, introducing an R230C mutation into the PH domain of SWAP70, which has been previously shown to abolish binding to the plasma membrane lipid PIP₃, [6] impaired neither the membrane localisation, nor the ability to drive these membrane extensions (Figure 2). The induction of filopodia caused by the expression of SWAP70-R is in line with previous studies over-expressing SWAP70 and confirms the cellular activity of this construct (Figure 2). The localisation of SWAP70-R was also studied in Swiss 3T3 fibroblasts, which have been shown to activate PI3K and RAC1 upon PDGF stimulation [28, 29]. We found that in these fibroblasts, evident enrichment of SWAP70-R on the plasma membrane was not seen in unstimulated cells, but a strong translocation of the protein was observed upon PDGF stimulation. This PDGF-stimulated translocation of SWAP70-R in fibroblasts appeared to be dependent upon binding to PIP₃ and/or PI(3,4)P₂ as it was not seen with the non-binding mutant SWAP70 R230C (Figure 3a and 3b). On the other hand, FRET-based analysis of the conformation of SWAP70 showed that even in unstimulated fibroblasts, a high-FRET sub-population of the protein was observed at the peripheral membrane of approximately half of these cells, which displayed little or no increase in abundance upon PDGF-stimulation, despite the observed bulk translocation of SWAP70-R protein to the cell membrane (Figure 3a). These experiments also confirm that tagging with fluorescent proteins and over-expression of SWAP70-R do not block PIP₃-dependent membrane recruitment.

SWAP70 undergoes conformational regulation at the leading edge of migratory U87MG cells

U87MG glioma cells are PTEN null, display several autocrine growth factor signalling loops and are highly motile [30-33]. When SWAP70-R was expressed in these motile cells, approximately half of the cells showed some focal peripheral enrichment of the

reporter, often in lamellipodia at the migratory leading edge (Figure 4). In the same experiments, the majority of cells showed peripheral regions with elevated FRET, which often did correspond to regions with evident enrichment of the probe but was also often observed in cells in which the probe itself appeared homogeneously distributed (Figure 4c). Very similar data were acquired in these experiments using the SWAP70 reporter for the R230C mutant (Figure 4). These data imply that the SWAP70 reporter protein is able to take up distinct conformations at the membrane but that these are not reliant upon binding of its PH domain to PIP₃. As SWAP70 has been reported to bind both to filamentous actin and to activated RAC1, we investigated the relationship between SWAP70 localisation and F-actin and the effect of inhibiting class I PI3K or RAC1 (Figure 5). In these experiments we used the pan class 1 PI3K inhibitor GDC0941 (also known as pictilisib) [34] and the RAC1 inhibitor EHT1864 [35]. In accordance with previous data, SWAP70 reporter showed a co-localisation with F-actin in Swiss3T3 fibroblasts, which was increased by the PIP₃-dependent membrane translocation of the protein (Figure 5a). A similar constitutive co-localisation of SWAP70-R with F-actin was observed in U87MG cells, which was suppressed by inhibition of either PI3K or RAC1 (Figure 5b-c). Similarly, SWAP70 R230C showed a level of co-localisation with F-actin, suggesting that the reduced co-localisation caused by PI3K inhibition is not simply caused by dramatic changes in cellular migration and to the cytoskeleton.

DISCUSSION

We have developed a FRET-based reporter to investigate the localised conformational regulation of SWAP70 in live cells. This shows that SWAP70 adopts a distinct high-FRET conformation in specific areas of the cell periphery. We also see that in polarised migratory cells, this conformation is enriched at the leading edge. The lack of correlation of this FRET signal with PI3K-dependent recruitment of SWAP70 onto the membrane implies that phosphoinositide-binding of the PH domain is neither necessary nor sufficient for the observed changes in SWAP70 conformation. On the other hand, we do not interpret this and the lack of impact of the R230C phosphoinositide binding mutation to mean that PI3K is not involved in the conformational activation of SWAP70. We suggest that the simplest explanation of the data is that binding to PIP₃ and/or PI(3,4)P₂ influences the localisation of SWAP70 on the plasma membrane, but that the key inputs to conformational control are the binding of other co-localised molecules, such as F-actin. We also suggest that the high FRET sub-population of SWAP70 revealed within these cells is likely to be the active, functionally significant pool capable of activating RAC1 and perhaps other GTPases. It is notable here that RAC1 and SWAP70 both appear to be quite abundant proteins (e.g. very approximately 500 000 and 50 000 molecules respectively per NIH3T3 or U2OS cell [36, 37]).

Our data are consistent with the previously demonstrated binding of SWAP70 to F-actin and RAC1 in addition to phosphoinositides and support a model in which SWAP70 mediates a positive feedback loop resulting in localised PI3K and RAC1

activity [17, 19-21, 38]. Mechanistically, this may also be linked to observations that some examples of receptor mediated activation of PI3K appear to require the actin cytoskeleton [39, 40]. Notably, it was observed in most of the experiments conducted during the study that approximately half of cells expressing SWAP70-R displayed a modestly higher FRET efficiency in the nucleus than the cytoplasm, despite appearing less abundant in the nucleus (e.g. Fig 3a lower). This implies that the nuclear and cytoplasmic localisations may influence the conformation of SWAP70, which resides in both compartments [41]. Although we have not followed up this observation, it is potentially related to the recognised differential regulation of RAC1 in the nucleus [42].

The enrichment of F-actin, PIP₃ and active RAC1 has previously been observed at the leading edge of adherent cells during their directed migration [24, 43-45]. Similarly, the PI3K-dependent recruitment of SWAP70 to the plasma membrane has been observed in growth factor stimulated fibroblasts and shown to be necessary for the organisation of F-actin within membrane ruffles in these cells [6]. However, the precise roles of SWAP70 and the many other GEF-family member capable of activating RAC1 in cell migration is unclear [18]. Our identification of a conformationally regulated pool of SWAP70 at the leading edge of migrating cells suggests that SWAP70 plays a part linking F-actin and PI3K to a stabilised active pool of RAC1 at this location. Our work also provides a new research tool, which is able to reveal conformational changes in SWAP70 occurring in live cells and which should help determine these detailed contributions of SWAP70 and other GEF family members in the control of RAC1 and cytoskeletal remodelling.

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FIGURE LEGENDS

Figure 1: SWAP70-R shows non-uniform FRET signal in U87MG cells.

(a) SWAP70 has four domains, an N-terminal EF hand like domain, a PIP₃ and/or PI(3,4)P₂ binding PH domain, a DH domain; and a C-terminal actin binding motif. The diagrams represent the design of the SWAP70-R (WT and PH domain mutant, R230C) FRET reporters with CFP variant mTurquoise at the N-terminus and YFP variant Venus at the C-terminus. (b) A diagram illustrates the structure of the FRET controls: C5V and CTV. C5V has a short linker of the length of 5 amino acids between the two fluorophores, which brings two fluorophores within the physical limits of FRET occurrence. Whereas, CTV has a 229 amino acid long linker between the two fluorophores, permitting a greater spatial separation of the fluorophores. (c) U87MG cells were transfected with the expression vectors encoding FRET controls or SWAP70-R. Control cells were transfected with expression vectors encoding CFP or YFP for the purpose of spectral bleed through corrections. 48 hours post-transfection cells were imaged using a wide-field microscope. Imaging in donor channel (ex. 430 nm and em. 470 nm) (*top panel*) shows the localization of the construct; and the FRET signal calculated with Image J/PFRET (*bottom panel*). The images shown are representative of four independent experiments with at least 6 cells analyzed per experiment. Scale bar = 15 μ m. Positive FRET control, C5V shows a uniform high FRET signal (~50%) and negative FRET control, CTV show a uniform low FRET signal (<10%) across the cell. SWAP70-R shows a non-uniform FRET signal (between 10% and 35%) with higher signal at the cell periphery compared to the interior of the cell.

Figure 2: SWAP70-R shows membrane localization and affects cellular morphology of U87MG cells independently of its ability to bind to PIP₃.

(a) U87MG human glioma cells were transfected with expression vectors encoding SWAP70-R (WT or R230C). Control cells were transfected with expression vectors encoding YFP. 24 hours post-transfection cells were imaged using a wide-field microscope in acceptor/YFP channel (ex. 500 nm and em. 530 nm). Scale bar = 15 μ m. Overexpression of SWAP70-R, both WT and R230C, lead to several distinct changes, examples of which are shown: membrane enrichment of SWAP70-R (*left panel*), extension of lamellipodia (*middle panel*) and an increased number of filopodia (*right panel*) compared to control cells. (b) Bar chart shows the percentage number of cells having ≥ 4 filopodia. The quantification is derived from three independent experiments and 10 randomly chosen fields of view for each construct per experiment. Error bars represent mean \pm SEM. Over-expression of both SWAP70-WT and SWAP70-R230C significantly increased the number of cells with

four or more filopodia compared to control cells (Student's T-test - $p < 10^{-5}$). The total number of cells counted was 488 for SWAP70, 455 for R230C and 421 for YFP.

Figure 3: SWAP70-R reveals a membrane-associated population of SWAP70 molecules with a distinct conformation, which is unaffected by an observed PH domain mediated membrane translocation

(a) Swiss-3T3 cells were transfected with expression vectors encoding SWAP70-R (WT or R230C). Serum starved cells were imaged live, for 2 minutes before treatment with PDGF (50 ng/mL) or vehicle for 10 minutes, with the presented images being captured immediately prior to stimulation and after 4 minutes. The image shows the localization of the reporter (left, blue) and the FRET efficiency (right, pink/purple) and are representative of four independent experiments. Scale bar = 15 μ m. (b) A bar chart showing the percentage of cells showing membrane translocation of the SWAP70-R upon treatment with vehicle or PDGF. Error bars represent the mean \pm SEM of four independent experiments, with 6-8 cells being imaged for each construct in each stimulation condition per experiment. Statistical significance was calculated using Student's t-test (unpaired, 2 tailed).

Figure 4: SWAP70-R shows a distinct conformation and enrichment at the leading edge of the motile U87MG cells which does not correlate strongly with its ability to bind PIP₃.

U87MG cells were transfected with expression vectors encoding SWAP70-R (WT and R230C). 48-hours post-transfection cells were imaged live (1 frame/min). White arrows show the direction of cell movement. Scale bar = 15 μ m. (a) SWAP70-R, both WT and R230C show high FRET signal on the leading edge of motile U87MG cells without or (b) with strong enrichment of the reporter in leading edge lamellipodia. (c) Quantification of the imaged cells in terms of motility, localization of SWAP70-R in the cell and FRET of SWAP70-R. The error bars represent the mean \pm SEM. (D) Quantification of the leading edge events of SWAP70-R in motile U87MG cells expressed as percentage number of motile U87MG cells showing reporter enrichment, high FRET or both on the leading edge of the cells. The error bars represent the mean \pm SEM. The images shown are representative of five experiments with 7-10 cells imaged for each construct per experiment.

Figure 5: SWAP70-R shows accumulation in actin rich sites of the cell. Cells were transfected with expression vectors encoding SWAP70-R (WT or R230C) or YFP as a control.

(a) Swiss-3T3 cells expressing YFP or SWAP70-R (WT or R230C) were treated with vehicle or PDGF (50ng/mL for 10 minutes) post 24 hours of serum starvation. Cells were fixed and then stained for endogenous F-actin using phalloidin alexa-633 after permeabilization (0.2% triton for 5 minutes). In PDGF stimulated Swiss-3T3 cells, SWAP70-R is found enriched on the membrane and actin rich sites (shown by arrows). (b) U87MG cells expressing YFP or SWAP70 (WT or R230C) were treated with DMSO or PI3K inhibitor (2 μ M GDC0941 for 20 minutes) or Rac1 inhibitor (10 μ M EHT1864 for 20 minutes). SWAP70-R (both WT and R230C) show localization in actin rich sites. Either PI3K or Rac1 inhibitor reduce/remove the localization of SWAP70 in actin rich sites. (c) Bar charts showing the percentage of cells showing localization of YFP or SWAP70-R (WT or R230C) in actin rich sites in U87MG cells upon treatment with DMSO or PI3K or Rac1 inhibitor (GDC0941 and EHT1864 respectively). The experiment was performed three times and error bars represent the mean \pm SEM. Statistical significance was calculated using Student's t-test. The total number of cells analyzed was for Venus: 27 for DMSO, 24 for GDC0941 and 24 for EHT1864. SWAP70-WT: 32 for DMSO, 26 for GDC0941 and 21 for EHT1864 and SWAP70-R230C: 30 for DMSO, 24 for GDC0941 and 28 for EHT1864.