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EVIDENCE FOR AN INTERACTION BETWEEN GOLLI AND STIM1 IN STORE-OPERATED CALCIUM ENTRY

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Golli and Stim1 interactions in store-operated calcium entry

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Myelin Basic Protein, Golli-MBP, STIM1, endoplasmic reticulum, calcium, store-operated calcium entry,

Abbreviations used:

ER, Endoplasmic reticulum; ER-PM junctions, Endoplasmic Reticulum-Plasma Membrane junctions; IP_3, inositol 1,4,5-trisphosphate; EYFP, enhanced yellow fluorescent protein; SOC, store operated calcium channel; SOCE, store operated calcium entry; MBP, myelin basic protein

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SYNOPSIS

Store operated Ca^{2+} channels (SOCs) are highly selective ion channels which are activated upon release of Ca^{2+} from intracellular stores to regulate a multitude of diverse cellular functions. It was recently reported that Golli-BG21, a member of the myelin basic protein (MBP) family of proteins, regulates store operated calcium entry (SOCE) in T-cells and oligodendrocyte precursor cells but the underlying mechanism for this regulation is unknown. Here, we have discovered Golli can directly interact with the ER Ca^{2+}-sensing protein, STIM1. Golli interacts with the C-terminal domain of STIM1 in both *in vitro* and *in vivo* binding assays and this interaction may be modulated by intracellular Ca^{2+} concentration. Golli also colocalises with full length STIM1 and Orai1 complexes in HeLa cells following store depletion. Overexpression of Golli reduces SOCE in HeLa cells but this inhibition is overcome by overexpressing STIM1. We therefore suggest that Golli binds to STIM1-Orai1 complexes to negatively regulate the activity of SOCs.

INTRODUCTION

Ca^{2+} signalling controls many cellular processes including cell growth, secretion and proliferation [1, 2]. Generation of Ca^{2+} signals in non-excitable cells involves rapid IP_3-dependent Ca^{2+} release from intracellular stores followed by external Ca^{2+} entry through store operated Ca^{2+} channels (SOCs) [3-5]. These coupled processes are regulated by two dynamic transmembrane proteins, the ER Ca^{2+} sensor protein STIM [6-10], and the plasma membrane protein Orai [9, 11]. STIM1 is a Ca^{2+} sensor within the ER which oligomerises to form aggregates or "puncta" in ER-PM junctions upon store depletion [7, 12]. Oligomerised STIM1 then binds to the plasma membrane Orai1 to activate Ca^{2+} influx [13, 14]. Mutational analysis of the STIM1 interacting domains revealed that the cytoplasmic C-terminus of STIM1 is necessary and sufficient for the activation of SOC influx and its associated current, I_{CRAC} [15]. More recently, a minimal region has been identified within the C-terminus of STIM1 which is required to interact with and activate Orai1 clustering and SOC influx [16-20].

A recent paper by Varnai et al suggested that STIM1 and Orai1 form part of a large macromolecular complex which may indicate the presence of other proteins within STIM1-Orai1 clusters [21]. One potential candidate for this is Golli protein, encoded by the myelin basic protein (MBP) gene. Golli proteins are alternative splice isoforms of the classic MBP proteins [22]. Unlike MBP, which is a major constituent of myelin in the nervous system, Golli proteins are expressed ubiquitously although their function is not well understood. The BG21 isoform of Golli (Golli-BG21) is expressed at high levels in T-cells [23] where it acts as a negative regulator of T-cell activation [22]. T-cells from Golli-deficient mice are hyperproliferative and exhibit increased IL-2 production [24]. It has been shown that this is due to enhanced SOC influx in Golli-deficient cells, suggesting that Golli is a negative regulator of SOCE [24]. Conversely, overexpression of Golli-BG21 in Jurkat T cells results in decreased SOC influx [24]. Golli-BG21 also regulates voltage-gated and store-operated calcium channels to mediate the migration and proliferation of oligodendrocyte precursor cells [25-27]. Interestingly, a fraction of Golli-BG21 is constitutively localised to lipid rafts at the

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plasma membrane and this plasma membrane association increases following T-cell stimulation [24]. Mutation of the N-terminal Golli myristoylation site required for plasma membrane association abolishes its ability to regulate Ca^{2+} influx [24]. Golli-BG21 does not affect the IP_3-dependent release of Ca^{2+} from internal stores but rather regulates store depletion induced Ca^{2+} entry itself [24]. It is not yet known how Golli-BG21 regulates SOC activity in T-cells, whether it is due to a direct effect on the channel itself or through the regulation of the signalling pathway responsible for SOC activation. Here we show that Golli-BG21 interacts with the SOC channel activator STIM1, and suggest that down-regulation of SOCE is the likely consequence of this interaction.

MATERIALS AND METHODS

Plasmid construction

All expression vectors were purchased from Clontech (France), except pGEX-6-P1 which was from GE Healthcare (Buckinghamshire, UK). The Golli-BG21-GFP plasmid was a gift from Dr Celia Campagnoni (UCLA, Los Angeles). For affinity chromatography and binding experiments, the C-terminal domain of STIM1 (STIM1-CT; amino acids M241-K685), the C- and N-terminal domains of Orai1 (Orai-CT (amino acids A267-A315) and Orai-NT (amino acids M1-L86) and the full length Golli-BG21 protein were subcloned into the pGEX-6-P1 vector using BamHI/SacII for STIM1-CT, and BamHI/XhoI for Golli and Orai. For Split-YFP experiments, STIM1-CT and Golli-BG21 were subcloned into the YFP-YN and YFP-YC vectors, respectively [28]. STIM1-CT was subcloned into pEYFP-N1 and YFP-YN using HindIII/SacII and Golli-BG21 was subcloned into pmCherry-N1 (a gift from Dr R.Y. Tsien, University of California, San Diego) and YFP-YC using XhoI/SacII for colocalisation experiments. Construction of STIM1-EYFP and mCherry-Orai1 has been described previously [29], as has construction of mCherry-Rit1 [30].

Affinity chromatography and MALDI-TOF

Extracts of bovine brain were prepared as described previously [31]. GST fusion proteins were expressed in *E. coli*, immobilised on glutathione cellulose beads (Bioline, London, UK) and incubated with 1 ml extracted cytosolic bovine brain proteins on a rotator for 2 h at $4^{o}C$. The beads were washed with Wash Buffer (25 mM Tris-HCl, 50 mM KCl, 1 mM DTT, pH 7.8) and proteins eluted eluted by boiling in Laemmli buffer. Eluted proteins were purified by methanol precipitation, denatured by boiling in SDS-buffer, and analysed by SDS-PAGE. Protein bands of interest were excised and subjected to in-gel tryptic digestion and MALDI-ToF mass spectrometry as previously described [32].

Binding assays

GST control or GST-Golli fusion proteins were immobilised on glutathione cellulose beads and incubated with STIM1-CT protein (2 µM) which had been PreScission-treated to cleave the GST tag. Samples were eluted by boiling in Laemmli buffer and

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analysed by Western blotting using an anti-STIM1 antibody (1:1000; ProSci Inc, California) and a HRP-linked anti-rabbit secondary antibody.

Cell culture and transfection

HeLa cells were maintained as described previously [29]. Cells were transfected with 1 µg of plasmid(s) of interest using GeneJuice (Merck Biosciences, Darmstadt, Germany) and cultured for 24h.

Bimolecular Fluorescence Complementation (BiFC) Assays and colocalisation studies

For BiFC assays, HeLa cells were transfected with Golli-BG21-YC and STIM1-CT-YN constructs either alone or together. For further control experiments, HeLa cells were transfected with the empty BiFC vectors (YFP-YC and YFP-YN), Golli-BG21-YC and YFP-YN, or STIM1-CT-YN and YFP-YC. Cells were treated with thapsigargin (2 µM) at room temperature for 90 min before imaging. For colocalisation studies, HeLa cells were cotransfected with mCherry-Orai1 together with Golli-BG21-YC/STIM1-CT-YN or STIM1-CT-YFP, STIM1-EYFP and mCherry-Golli, or triply transfected with STIM1-EYFP, mCherry-Orai1 and Golli-BG21-GFP and imaged 24h post-transfection cells.

Ca^{2+} Measurements

Cells grown on glass-bottom dishes were placed in standard buffer (NaCl (140 mM), KCl (4.7 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1.13 mM), glucose (10 mM), Hepes (10 mM), pH 7.3) and loaded with Fluo-4 AM (5 µM) for 30 min at RT before washing in calcium-free standard buffer. Ca^{2+} imaging was carried out using a Leica AOBS SP2 microscope (Leica Microsystems AG, Wetzlar, Germany). HeLa cells expressing STIM1-EYFP, Golli-mCherry or a combination of both were pretreated with 2 µM thapsigargin (Calbiochem, California, USA) in the absence of extracellular Ca^{2+} for 10 min to deplete stores. 2 mM Ca^{2+} was added to activate SOCE [30].

Quantification of STIM1 puncta

Quantification of STIM1-EYFP puncta was performed as described previously [30]. Briefly, puncta were selected as spots of high fluorescence intensity ranging from approximately 0.5 – 1.0 µm in diameter. Accuracy of puncta quantification was verified by independent blind counting.

Quantification of Golli-mCherry fluorescence

To determine the extent of Golli-mCherry fluorescence in cells over-expressing STIM1-EYFP versus cells that do not over-express STIM1, regions of interest were drawn around the outside of each cell. Fluorescence values were calculated on the basis of the mean fluorescence per pixel within the cell rather than total fluorescence to avoid problems arising from variations in the size of the selected regions of interest.

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RESULTS

To search for novel binding partners for STIM1 and Orai1, we performed pull-down experiments on immobilised cytosolic domains of the STIM1 and Orai1 GST-fusion proteins (GST-STIM-CT (~80 kD), GST-Orai1-CT (~34.5 kD) and GST-Orai1-NT (~33 kD)) using bovine brain cytosol (Figure 1A). MALDI-ToF mass spectrometry analysis of unique protein bands from the pull-down identified myelin basic protein (MBP; 8 matching peptides with 39% sequence coverage), as a protein of approximately 25 kD in size which bound to the C-terminus of STIM1. Western blotting using an anti-MBP antibody confirmed that MBP bound specifically and efficiently to STIM1 and also bound to the N-terminus of Orai1 (Figure 1B). Note that a similar sized polypeptide was present in the control GST pull-down sample (Figure 1A). The identity of this polypeptide was not established but it was not recognised by the anti-MBP antibody (Figure 1B). The finding of MBP binding was surprising, since classic MBP itself is required for central nervous system myelination and has not been implicated in the activity of store operated calcium channels. The Golli family of proteins are alternatively-spliced variants of classical MBPs which have been shown to mediate signal transduction in T cells through the negative regulation of SOCs [24]. Since Golli proteins contain MBP epitopes, we hypothesised that Golli may interact with STIM1 to exert its effect on SOCE. We assessed whether the C-terminus of STIM1 interacts directly with Golli using an *in vitro* binding assay. A recombinant GST-Golli-BG21 fusion protein (~57.5 kD) was expressed in *E*. coli and immobilised on glutathione affinity resin. Immobilised GST-Golli-BG21 or GST control protein (~26 kD) was incubated with prescission-cleaved STIM1-CT (~54 kD). Western blotting analysis revealed that STIM1-CT bound specifically to the GST-Golli-BG21 protein but not to the GST control (Figure 1D), confirming that the C-terminus of STIM1 does indeed interact with Golli *in vitro*. Note that in these experiments the glutathione beads were incubated with levels of GST or GST-Golli-BG21 that resulted in a 1.4-fold higher level of GST so that lack of binding of STIM1-CT to GST loaded beads was not a consequence of the relative GST concentration. In similar experiments binding of Orai-NT to GST-Golli-BG21 could not be detected (data not shown).

To further investigate the possible interaction of STIM1 and Golli-BG21 in a cellular context, we expressed these two proteins in HeLa cells using a Bimolecular Fluorescence Complementation (BiFC) Assay [33]. This BiFC system involved the fusion of the complementary fragments of EYFP to two proteins of interest. If these proteins interact the EYFP fragments come close enough to fold and form a functional fluorescent EYFP protein [28]. In these experiments, complementary fragments of EYFP (YC and YN) were fused to the C-termini of the STIM1-CT (STIM1-CT-YN) and Golli (Golli-BG21-YC) proteins and expressed in HeLa cells along with a previously described mCherry-tagged Rit1 tail protein (mCherry-Rit1; [30]) as a marker to identify transfected cells. No detectable fluorescence was observed in untreated cells (Figure 2A).Transfected cells were treated with thapsigargin for 60 – 90 min at room temperature in 2mM external Ca^{2+} before imaging with a confocal

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microscope. EYFP fluorescence was detected at the plasma membrane in treated cells expressing STIM1-CT-YN and Golli-BG21-YC ~90min following the addition of thapsigargin (Figure 2B) which is consistent with the time-course described for the refolding of EYFP at room temperature [33]. EYFP fluorescence was distributed throughout the plasma membrane but no puncta were visible. The uniform plasma membrane EYFP fluorescence observed here is consistent with the previous finding that although STIM1-CT expression gives constitutive activation of the I_{crac} current, it does not form puncta at the plasma membrane [34] since it requires an ER localisation to oligomerise prior to translocation to ER-PM junctions [34] [35]. We also observed that STIM1-CT-EYFP colocalises with an mCherry-tagged Orai1 protein (mCherry-Orai1) at the plasma membrane in HeLa cells but does not form visible puncta (data not shown). No detectable EYFP fluorescence was observed in thapsigargin treated cells expressing either STIM1-YN or Golli-BG21-YC fusion protein alone (Figure 2C & 2D) or along with the appropriate complementary fragment of YFP (Figure 2E & 2F). Similarly, co-expression of both EYFP fragments failed to produce a fluorescent signal and served as a negative control (Figure 2G). There are two possibilities to explain why EYFP fluorescence was observed at the plasma membrane only following thapsigargin treatment. Firstly, STIM1-CT-YN may associate with endogenous STIM1 puncta enabling it to interact with Golli-BG21-YC at the plasma membrane. It is also possible that a change in cytosolic Ca^{2+} concentration may be required to modulate an interaction between STIM1-CT-YN and Golli-BG21-YC. To investigate this, HeLa cells expressing both constructs were treated with 100 µM histamine, which should induce transient oscillations in cytoplasmic Ca^{2+}, in place of thapsigargin. Plasmalemmal EYFP fluorescence formation was observed (Figure 2H) even though full length STIM1-EYFP did not reveal substantial sustained puncta formation under these conditions (data not shown) suggesting that an increase in cytosolic Ca^{2+} from intracellular stores modulates the interaction between STIM1-CT and Golli-BG21. Plasma membrane EYFP fluorescence was also observed in cells which were not cotransfected with mCherry-Rit1 (data not shown), eliminating the possibility that the transfection marker was targeting the BiFC constructs to the plasma membrane. The emission spectrum for the fluorescence observed with STIM1-YN/Golli-BG21-YC was close to that of EYFP (Figure 2I), confirming that the fluorescence observed was due to formation of functional EYFP. The BiFC assay was also used to determine whether Golli interacts with full length STIM1-YN or Orai1-YN. However, EYFP fluorescence was not observed in cells transfected with Golli-YC and Orai1-YN or full length STIM1-YN which had been treated with thapsigargin for 90 mins (data not shown).

Interestingly, when mCherry-Orai1 was coexpressed with STIM1-CT-YN/Golli-BG21-YC in HeLa cells, the EYFP fluorescence clearly overlapped with the mCherry-Orai1 signal (Figure 3A). Furthermore, a Golli-BG21-GFP fusion colocalised with both mCherry-Orai1 and STIM1-CT-EYFP in HeLa cells (Figure 3B and inlay). This led us to examine whether Golli colocalises with full length STIM1 and Orai1 in puncta, thereby placing the protein in a position to regulate the activity of these proteins. Golli overexpression has been shown to decrease SOCE following store

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depletion in Jurkat T cells [24]. If this occurred through an interaction with STIM1, the Golli protein would either colocalise with STIM1 in puncta or would prevent STIM1 puncta formation. To test this, full length STIM1-EYFP and a Golli-BG21-mCherry fusion protein were expressed in HeLa cells. No colocalisation between STIM1-EYFP and Golli-BG21-mCherry was observed in untreated HeLa cells, where STIM1-EYFP expression was reticular and Golli-BG21-mCherry was distributed throughout the plasma membrane as previously observed [24] (Figure 3C). However, when cells were treated with thapsigargin for 10 min before imaging to induce STIM1 puncta formation, Golli-mCherry expression was observed predominantly at the plasma membrane where STIM1-EYFP puncta accumulated but was also seen to colocalise with STIM1-EYFP in a subset of puncta (Figure 3D and inlay). We also examined whether Golli-GFP colocalised with punctate STIM1-EYFP and mCherry-Orai1 complexes in HeLa cells after thapsigargin treatment. Indeed it appeared that all three proteins colocalised to the same punctate structures (Figure 3E and inlay). We did not see any change in the number of STIM1-EYFP puncta formed in thapsigargin-treated cells overexpressing Golli-mCherry compared with control cells expressing STIM1-EYFP alone (Figure 3F) suggesting that Golli does not affect the formation of STIM1 puncta *per se*. The targeting of both proteins to the same punctate structures suggests that Golli and full length STIM1 interact and that Golli may regulate store operated calcium entry through a direct interaction with STIM1 in puncta.

To determine whether Golli affects SOCE in HeLa cells, we transfected cells with Golli-mCherry or STIM1-EYFP alone or in combination and loaded cells with the cytosolic Ca^{2+} indicator, Fluo-4 AM. Stores were depleted with thapsigargin in Ca^{2+}-free medium for approximately 10 min and Ca^{2+} influx was measured upon readdition of Ca^{2+} to the external solution. Untransfected cells in the same field of view as transfected cells were used as internal controls. Golli-expressing cells exhibited a reduced rate and extent of Ca^{2+} influx (Figure 4A, blue trace) compared with influx in untransfected control cells (Figure 4A, green trace) which was statistically significant ($p<0.0001$) at the 750 second time-point. This is consistent with previous findings that Golli-deficient T-cells show enhanced SOCE and that over-expression of Golli-BG21 inhibits SOCE [24], and suggests that Golli negatively regulates SOCE. In STIM1-overexpressing cells there was an increase in the initial rate of SOCE when compared with untransfected cells (Figure 4A, red trace), which we have observed previously [30]. Interestingly, over-expression of STIM1 abolished the inhibitory effect of Golli-BG21(Figure 4A, black trace) when compared with STIM1-overexpressing cells. This was not due to differences in the expression of Golli since the level of Golli-BG21-mCherry fluorescence was similar in cells expressing Golli-BG21-mCherry alone or in combination with STIM1-EYFP (Figure 4B). It is possible, therefore, that high levels of STIM1 protein expression can directly overcome the inhibition of SOCE by Golli-BG21.

DISCUSSION

Previous studies identified Golli-BG21 as a regulator of SOCE. This has been particularly well established for T-cells where knock out of Golli increased SOCE and

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overexpression of Golli-BG21 decreased SOCE [24]. It was not clear, however, from these studies whether Golli has a direct effect on the SOC channel itself or another protein or pathway involved in the activation of SOCE. In this study, we suggest that Golli directly interacts with the master SOCE regulator, STIM1, to reduce SOCE. The C-terminal domain of STIM1 successfully bound to Golli in GST binding assays and BiFC assays. We have not mapped the STIM1-interacting domain within Golli but it is possible that this is an epitope which is shared with the classical MBP protein since the C-terminus of STIM1 binds to MBP from bovine brain extract in pulldown assays. We have shown here using a BiFC assay that the interaction between Golli-BG21 and the C-terminus of STIM1 can be detected in cells after stimulation by both thapsigargin and histamine treatment. Both of these compounds should produce changes in cytosolic Ca^{2+} levels but, unlike thapsigargin, histamine elicits transient Ca^{2+} oscillations in HeLa cells [36, 37]. Since the formation of STIM1-EYFP puncta was not observed under conditions where histamine stimulated BiFC fluorescence between STIM1-YN and Golli-YC, it is possible that it is the modulation of the cytosolic Ca^{2+} concentration by both thapsigargin and histamine rather than sustained translocation of endogenous STIM1 that induces an interaction between the C-terminus of STIM1 and Golli-BG21. The BiFC assay was used in this study since it is a more robust method for investigating protein interactions in live cells than other techniques such as FRET. It is important to note however, that the BiFC assay is not reflective of the kinetics of the interaction, since binding between the YC and YN fragments of EYFP is essentially irreversible and refolding of the full EYFP protein and the reconstitution of fluorescence requires some time [33], in this case, up to 90 minutes. Irrespective of the lack of information from these experiments on the kinetics of STIM1/Golli-BG21 interaction, this assay provides the most convincing evidence of a direct protein-protein interaction in living cells.

No fluorescence complementation was observed in cells expressing Golli-YC and Orai1-YN, or Golli-YC and full length STIM1-YN (data not shown). EYFP fluorescence was also absent in cells expressing Orai1-YC and STIM1-CT-YN (data not shown), even though the STIM1 and Orai1 proteins are known to interact [16, 34, 38]. There are several reasons why this may have occurred. Firstly, fusion of an EYFP fragment to a protein may alter the structure of the protein, thereby abolishing an interaction domain within the protein of interest [39]. Secondly, the arrangement of the BiFC fusion proteins within a protein complex may sterically hinder an interaction between the complementary EYFP fragments and prevent the formation of functional EYFP [39]. The lack of EYFP fluorescence between Golli-YC/Orai1-YN and Golli-YC/full length STIM1-YN proteins is therefore not conclusive evidence for the absence of an interaction between these protein pairs. We also could not confirm the potential interaction of Orai-NT to GST-Golli-BG21 seen in the initial GST pull down from brain extract in a direct binding assay with recombinant proteins.

Despite the large amount of recent research into the activation of SOCs, very few interacting partners have been found for STIM1 and Orai1. One recent study has identified a novel EF-hand containing protein, CRACR2A, which binds to both STIM1 and Orai1 to stabilise their interaction, thereby enhancing SOCE in T-cells

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[40]. Additionally, little is known about the Ca^{2+}-dependent inactivation of these channels. One recent study reported that both calmodulin and a short negatively charged region within the STIM1 C-terminus can bind to Orai1 to inactivate SOCE [41]. Interestingly, in our studies, Golli-BG21 colocalised with STIM1 and Orai1 complexes after store depletion. The ability of Golli-BG21 to bind STIM1 puncta in a Ca^{2+}-dependent manner may be key to its regulation of SOCE. It is possible that Golli-BG21 binds to STIM1-Orai1 complexes when Ca^{2+} levels are already high to reduce the influx of Ca^{2+} into the cell and perhaps may be a further contributor to the Ca^{2+}-dependent inactivation of SOCs. When STIM1 was overexpressed with Golli-BG21, the inhibitory effect of Golli-BG21 on SOCE was abolished. This was not due to variation in the expression levels of Golli-BG21achieved in the presence and absence of STIM1, suggesting that an interaction between STIM1 and Golli-BG21 overcomes the ability of Golli to reduce SOCE.

To date, studies on MBP and Golli and their regulation of Ca^{2+} channels have focussed exclusively on oligodendrocyte precursor cells and T-cells. Of the three Golli isoforms cloned, the BG21 isoform of Golli is the most widely expressed in many different tissues other than nervous tissues, including the heart, kidney, spleen and lung [23] although its function in these tissues has not been elucidated. The widespread distribution of Golli-BG21 suggests that it could be part of a general mechanism for the regulation of SOCE across many tissue types. Hence, we propose that Golli-BG21 functions to regulate SOCE via a direct interaction with STIM1 but the exact molecular mechanism underlying this interaction has yet to be defined.

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FIGURE LEGENDS

Figure 1. Golli binds to the C-terminus of STIM1. (**A**) Coomassie blue stained gel of eluates from GST, GST-Orai1-CT, GST-Orai1-NT and GST-STIM1-CT pulldown

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assays. GST columns were run in parallel with GST-Orai1-CT, GST-Orai1-NT and GST-STIM1-CT and incubated with bovine brain cytosol extract. Samples were eluted by boiling in Laemmli buffer. Eluted proteins were separated by SDS-PAGE. Subsequently, proteins were excised from gels and analysed by MALDI-ToF. Myelin Basic protein (arrow) was identified as a GST-STIM1-CT binding partner. (**B**) Western blotting analysis of eluates from GST, GST-Orai1-CT, GST-Orai1-NT and GST-STIM1-CT pulldown assays. Membranes were blotted with anti-STIM1-CT antibody (ProSci; 1:10,000) (**C)** Purified STIM1-CT binding to GST-Golli. GST-Golli and GST-STIM1-CT fusion proteins were purified using glutathione cellulose beads. GST-STIM1-CT was cleaved with prescission TM protease to remove the GST tag. Glutathione cellulose beads containing equal amounts of GST or GST-Golli were incubated with 2 µM STIM1-CT. Proteins were eluted in wash buffer containing NaCl (1 mM), methanol precipitated and boiled in Laemmli buffer. Samples were separated by SDS-PAGE and analysed by Western Blotting.

Figure 2. STIM1-CT-YN and Golli-BG21-YC fusion proteins reconstitute EYFP fluorescence at the plasma membrane. HeLa cells were cotransfected with mCherry-Rit1 as a transfection marker. 24h post-transfection, cells were treated with 2 µM thapsigargin for 90 min and imaged using a confocal microscope, except in (**A**) where cells were imaged without treatment. (**A**) Co-expression of Golli-YC/STIM1-CT-YN in untreated cells does not result in the formation of EYFP; n = 21 cells. (**B**) Co-expression of Golli-YC/STIM1-CT-YN results in reconstitution of EYFP fluorescence at the plasma membrane when cells were treated with thapsigargin; n = 35 cells. Expression of either Golli-YC alone (**C**; n = 12 cells) or with YN (**D**; n = 20 cells) or STIM1-CT-YN alone (**E**; n = 27 cells) or with YC (**F**; n = 18 cells) fails to restore EYFP fluorescence in thapsigargin treated cells. Similarly, co-expression of the YC and YN proteins gives no EYFP fluorescence following thapsigargin treatment (**G**). Treatment of cells with 100 µM histamine in place of thapsigargin also reconstitutes EYFP fluorescence, n = 27 cells (**H**). EYFP fluorescence from (**B**) was recorded using the Lambda scan mode. Fluorescence intensity after 453 nm excitation was recorded in 5 nm intervals from 510 nm to 590 nm. The emission spectrum was similar to that of EYFP (**I**). Scale bars = 10 µm.

Figure 3. STIM1, Orai1 and Golli colocalise in HeLa cells. (A) HeLa cells were cotransfected with STIM1-CT-YN/Golli-YC and mCherry-Orai1. 24h post-transfection, cells were treated with 2 µM thapsigargin for 90 min and imaged using a confocal microscope. Reconstituted EYFP fluorescence from STIM1-CT-YN/Golli-YC colocalises with mCherry-Orai1. (**B)** STIM1-CT-EYFP, mCherry-Orai1 and Golli-BG21-GFP show colocalisation in HeLa cells (the enlarged insert is taken from top left of cell)). (**C**) Full length STIM1-EYFP does not colocalise with Golli-BG21-mCherry in untreated cells. (D) STIM1-EYFP puncta show colocalisation with Golli-BG21-mCherry in thapsigargin-treated HeLa cells (the enlarged insert is taken from

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top of cell)). (**E**) Full length STIM1-EYFP, mCherry-Orai1 and Golli-BG21-GFP show colocalisation in puncta following thapsigargin treatment in HeLa cells (the enlarged insert is taken from the middle left side of cell). (**F**) Cells expressing STIM1-EYFP alone (n=43) or in combination with Golli-BG21-mCherry (n=19) were analysed and the number of puncta formed following thapsigargin treatment counted and expressed as mean per cell ±SEM . There was no significant difference in the number of STIM1 puncta formed in the presence or absence of Golli-BG21-mCherry. Scale bars = 10 µm.

Figure 4. Overexpression of Golli reduces store-operated Ca^{2+} influx but this is reversed by overexpression of STIM1. HeLa cells were transfected with Golli-BG21-mCherry, STIM1-EYFP alone or together. 24h post-transfection, cells were loaded with Fluo-4 (5 µM) at room temperature for 30 min. Cells were treated with 2 µM thapsigargin to deplete the stores followed by the readdition of 2 mM Ca^{2+} to the external solution. Changes in $[Ca^{2+}]_i$ were measured and compared in transfected and untransfected cells. The data shown are means ± SEM. For untransfected cells, n = 154 cells. For Golli-BG21-mCherry transfected cells, n = 63 cells. For STIM1-EYFP transfected cells, n = 36 cells. For Golli-BG21-mCherry and STIM1-EYFP transfected cells, n = 55 cells. (**B**) Golli-BG21-mCherry fluorescence was quantified in cells expressing Golli-BG21-mCherry alone (n = 175) or together with STIM1-EYFP (n = 128). Expression levels of Golli-BG21-mCherry were similar in both conditions.

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Figure 1

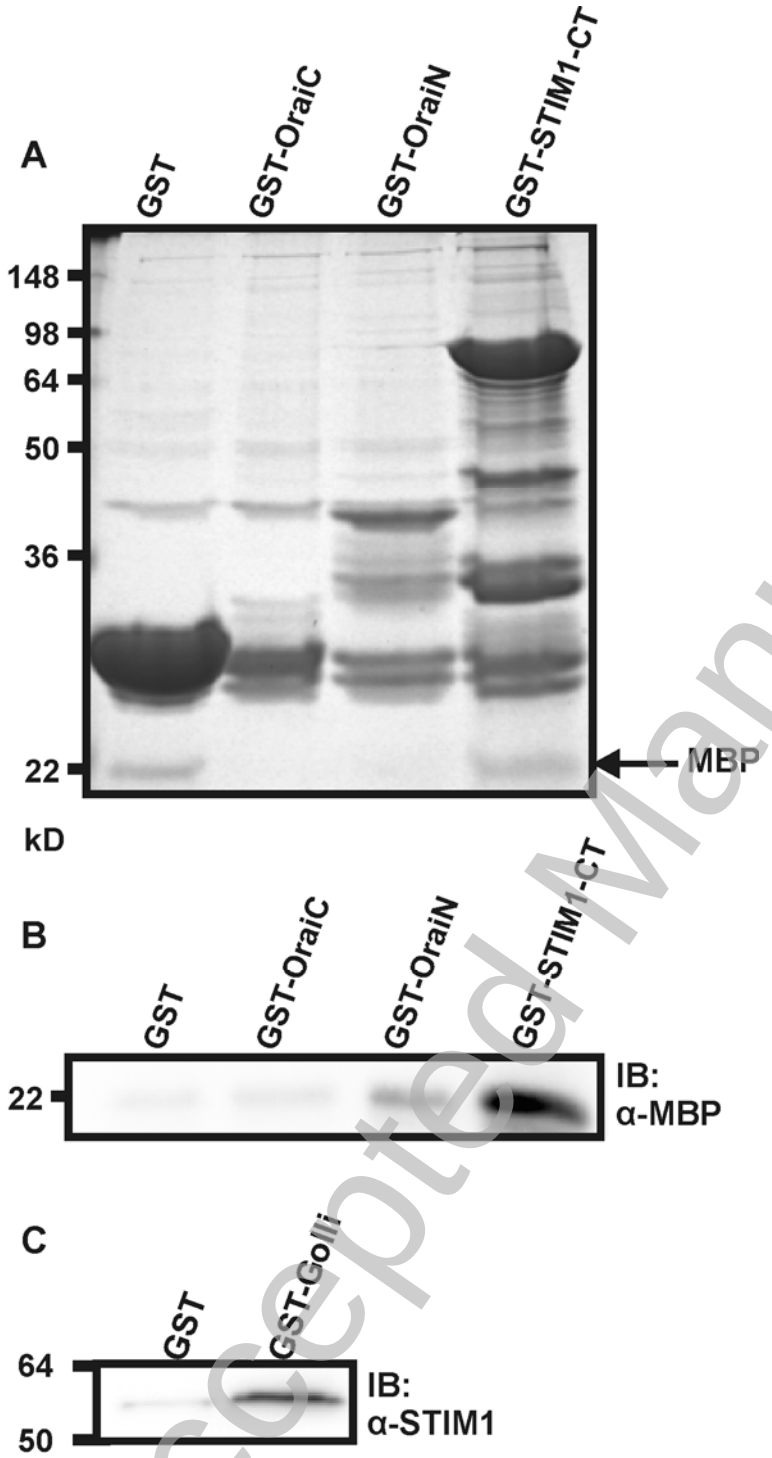

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Figure 2

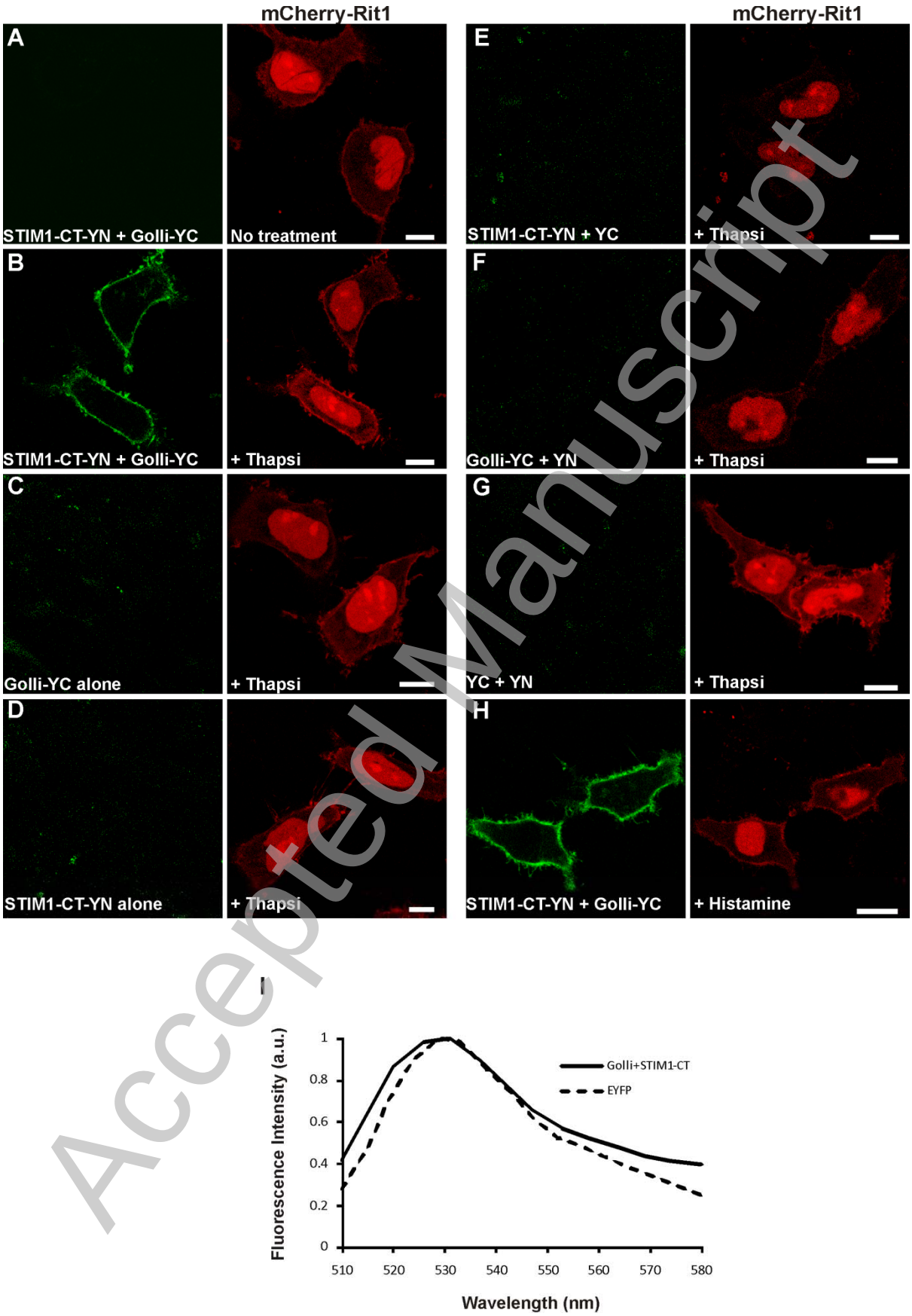

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Figure 3

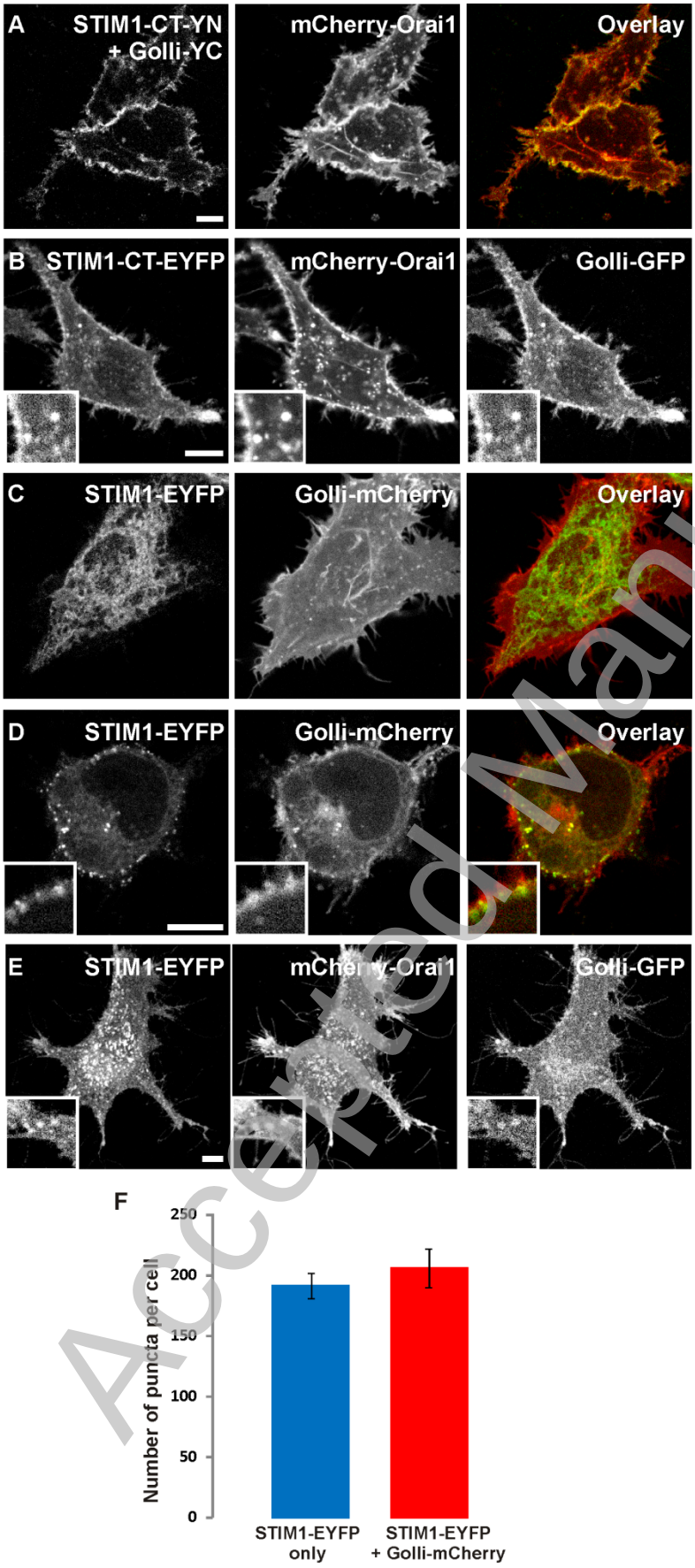

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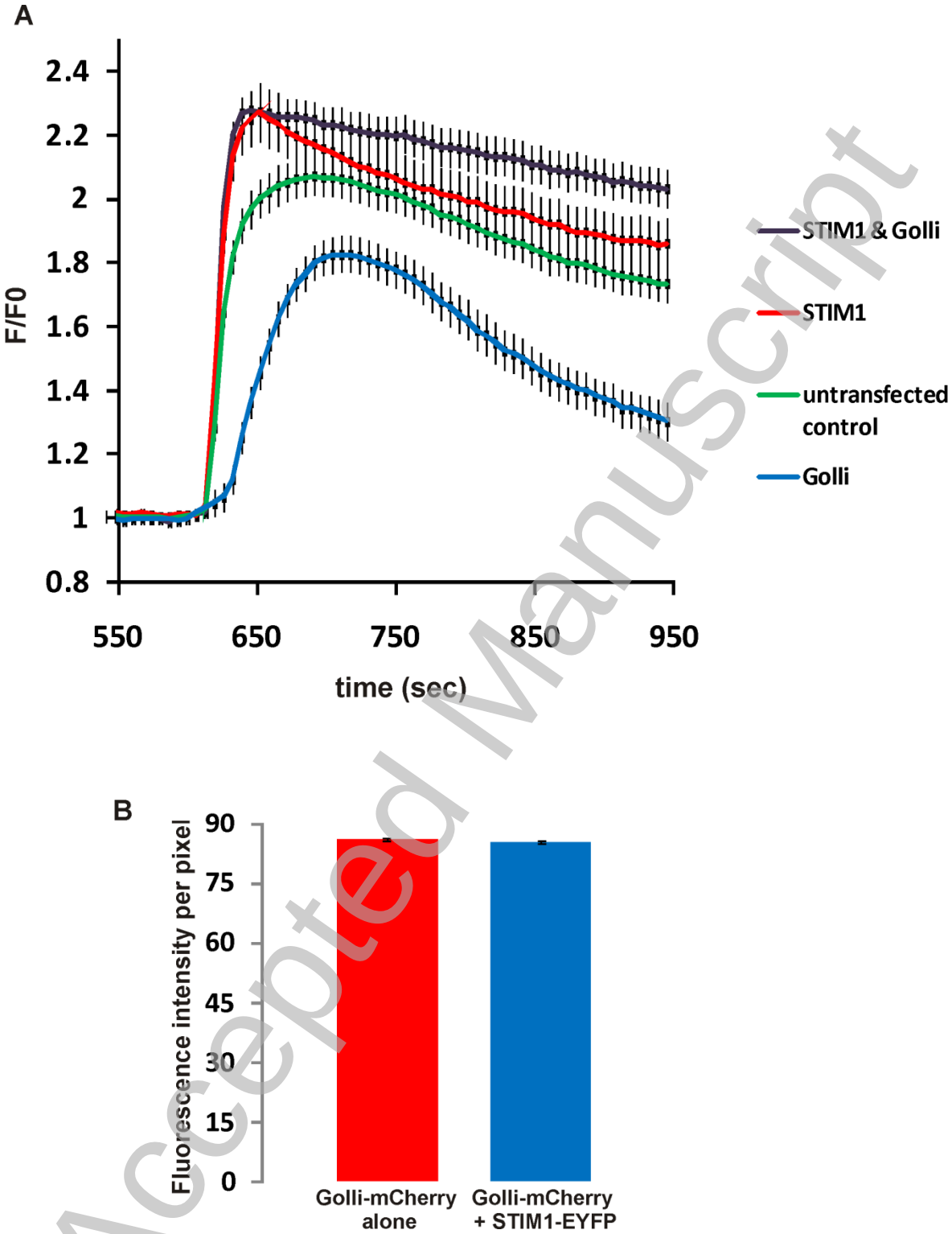
Figure 4



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