

RIM1 α SUMOylation Is Required for Fast Synaptic Vesicle Exocytosis

Fatima Girach,^{1,2} Tim J. Craig,^{1,2} Daniel L. Rocca,¹ and Jeremy M. Henley^{1,*}

¹Department of Biochemistry, School of Medical Sciences, University Walk, University of Bristol, Bristol BS8 1TD, UK

²These authors contributed equally to this work

*Correspondence: anjmh@bristol.ac.uk

<http://dx.doi.org/10.1016/j.celrep.2013.10.039>

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

SUMMARY

The rapid, activity-dependent quantal presynaptic release of neurotransmitter is vital for brain function. The complex process of vesicle priming, fusion, and retrieval is very precisely controlled and requires the spatiotemporal coordination of multiple protein-protein interactions. Here, we show that posttranslational modification of the active zone protein Rab3-interacting molecule 1 α (RIM1 α) by the small ubiquitin-like modifier 1 (SUMO-1) functions as a molecular switch to direct these interactions and is essential for fast synaptic vesicle exocytosis. RIM1 α SUMOylation at lysine residue K502 facilitates the clustering of Ca_v2.1 calcium channels and enhances the Ca²⁺ influx necessary for vesicular release, whereas non-SUMOylated RIM1 α participates in the docking/priming of synaptic vesicles and maintenance of active zone structure. These results demonstrate that SUMOylation of RIM1 α is a key determinant of rapid, synchronous neurotransmitter release, and the SUMO-mediated “switching” of RIM1 α between binding proteins provides insight into the mechanisms underpinning synaptic function and dysfunction.

INTRODUCTION

Activity-dependent neurotransmitter release is mediated by the Ca²⁺-dependent fusion of synaptic vesicles at the active zone of the presynaptic membrane (Südhof and Rizo, 2011). Rab3-interacting molecule 1 α (RIM1 α) interacts, either directly or indirectly, with most active zone proteins (Calakos et al., 2004) and is crucial to active zone function (Wang et al., 1997). More specifically, it participates in vesicle priming via interactions with Munc13-1 (Deng et al., 2011; Koushika et al., 2001), Ca²⁺ channel clustering near release sites (Coppola et al., 2001; Kaeser et al., 2011), and synaptic plasticity, including presynaptic LTP (Castillo et al., 2002) and homeostatic plasticity (Müller et al., 2012). Furthermore, interactions between RIM1 α and Rab3a (Lonart, 2002; Wang et al., 1997) and synaptotagmin (Coppola et al., 2001) suggest roles in vesicle docking

and Ca²⁺ triggering of exocytosis, respectively. Thus, RIM1 α acts as a hub in a diverse range of functions, but it is unknown how RIM1 α binding to its multiple interacting proteins is regulated.

Posttranslational protein modification by SUMOylation is a fundamentally important regulatory mechanism in nearly all cell pathways (Hay, 2005). Small ubiquitin-like modifier 1 (SUMO-1) is a 97-residue peptide that attaches to proteins via an isopeptide bond to the primary amine groups of lysine residues. This covalent attachment is catalyzed by the E2 enzyme Ubc9, which binds to the substrate protein, and is removed by SUMO-specific proteases (SENPs) (Flotho and Melchior, 2013). In neurons, SUMOylation participates in the regulation of synapse formation (Shalizi et al., 2006), neurotransmitter receptor trafficking, synaptic plasticity (Martin et al., 2007; Craig et al., 2012; Jaafari et al., 2013), and presynaptic neurotransmitter release (Feligioni et al., 2009). However, most of the SUMO substrate proteins mediating these effects are unknown.

In this study, we identify RIM1 α as a synaptic SUMO substrate. Abrogation of RIM1 α SUMOylation leads to severe defects in action potential (AP)-evoked presynaptic exocytosis and Ca²⁺ entry, but not vesicle docking or priming. We show that inhibition of RIM1 α SUMOylation dramatically reduces its PDZ domain interaction with Ca_v2.1 and suggest that RIM1 α SUMOylation serves to delineate the many different functions of this protein.

RESULTS AND DISCUSSION

RIM1 Is a Neuronal SUMO Substrate

To identify neuronal SUMOylation substrates, we used GST-tagged Ubc9 to affinity purify binding proteins from extracts of rat cortical neurons. Mass spectrometry and western blotting showed that RIM interacts with Ubc9 (Figure 1A). Anti-SUMO-1 antibody immunoprecipitated a RIM1/RIM2-reactive band of the correct predicted molecular weight, which was protected by NEM, which inhibits SENP-mediated deSUMOylation (Figures 1B and 1C). Consistent with RIM being a SUMO substrate, RIM1/RIM2 and SUMO-1 show extensive colocalization (Mander's M1 colocalization coefficient of 0.6870 \pm 0.01, where M1 represents the amount of SUMO-1 fluorescence that overlaps RIM1/RIM2 fluorescence, n = 35) in the processes of hippocampal neurons (Figure 1D). This colocalization shows that SUMO-1

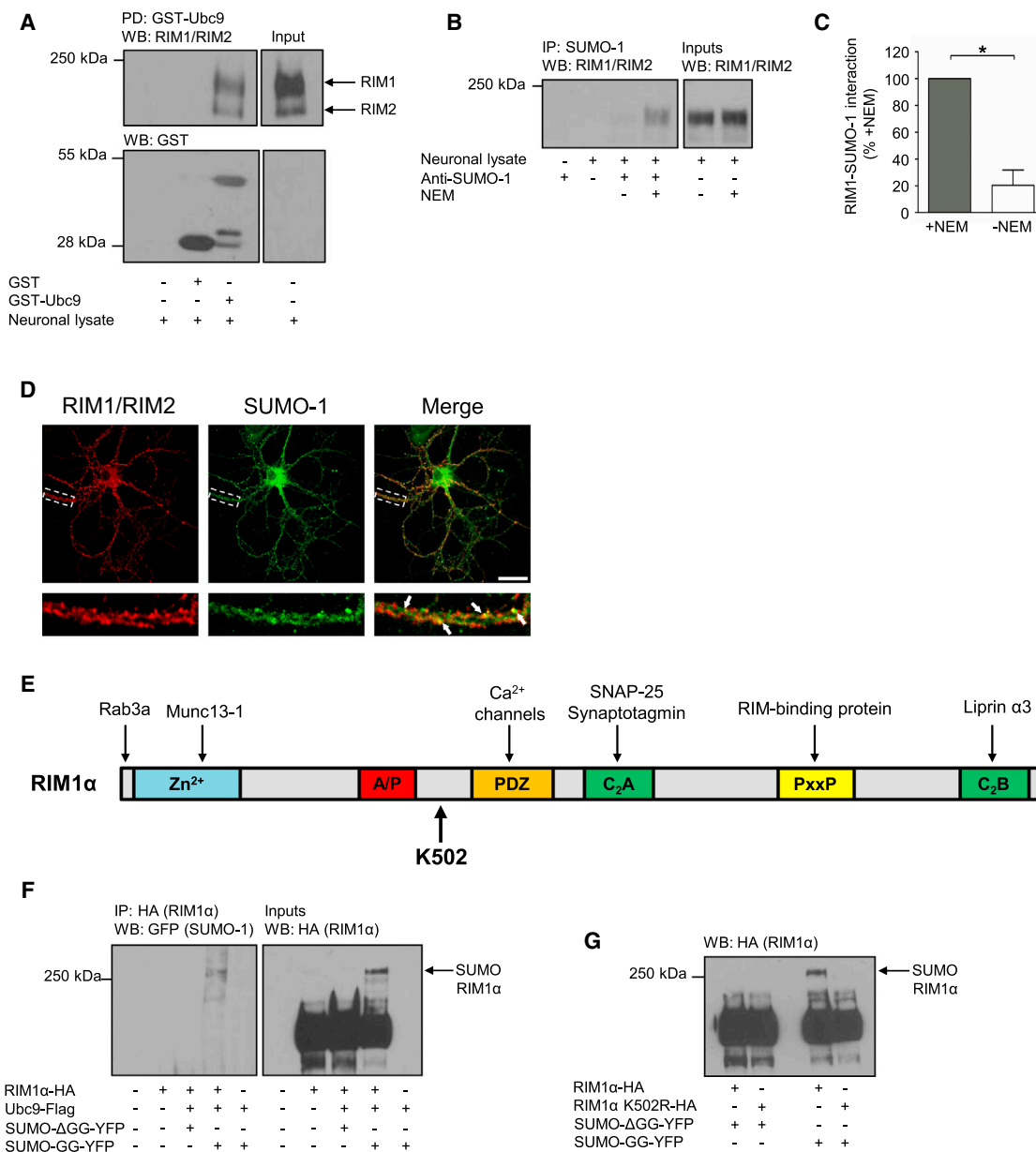


Figure 1. RIM1 Is a SUMO Substrate in Neurons

(A) GST-Ubc9 pull-down (PD) for RIM1/RIM2 from rat cortical lysate is shown. WB, western blot.

(B) Coimmunoprecipitation of RIM1/RIM2 with SUMO-1 from cortical neurons is presented. IP, immunoprecipitation.

(C) Quantification of (B) is expressed as the percent (%) RIM1/RIM2 immunoprecipitated in the presence of NEM ($n = 3$). * $p < 0.05$ (Student's t test). Data are represented as mean \pm SEM.

(D) Representative images present RIM1/RIM2 (red) and SUMO-1 (green) immunostaining in hippocampal neurons. Panels below show magnification of the area in the dashed boxes. Arrows highlight colocalization. The scale bar represents 20 μm .

(E) Schematic shows significant features and binding sites in RIM1 α .

(F) Coimmunoprecipitation of SUMOylated RIM1 α in HEK293T cells is presented. Samples immunoprecipitated with HA (RIM1 α) were blotted for GFP (SUMO). Representative of four blots is shown. SUMO- Δ GG is a nonconjugatable form of SUMO used as a negative control (cf. conjugatable SUMO-GG).

(G) Western blot shows that K502R mutation abolishes RIM1 α SUMOylation in HEK293T cells.

See also Figure S1.

is present in the presynapse and thus has the potential to influence the presynaptic functions of RIM1/RIM2, although it is likely that there are many presynaptic substrates.

In subsequent experiments, we focused on the RIM1 α isoform because of the higher abundance and well-characterized presynaptic role (Figure 1A; Schoch et al., 2006). RIM1 α is a

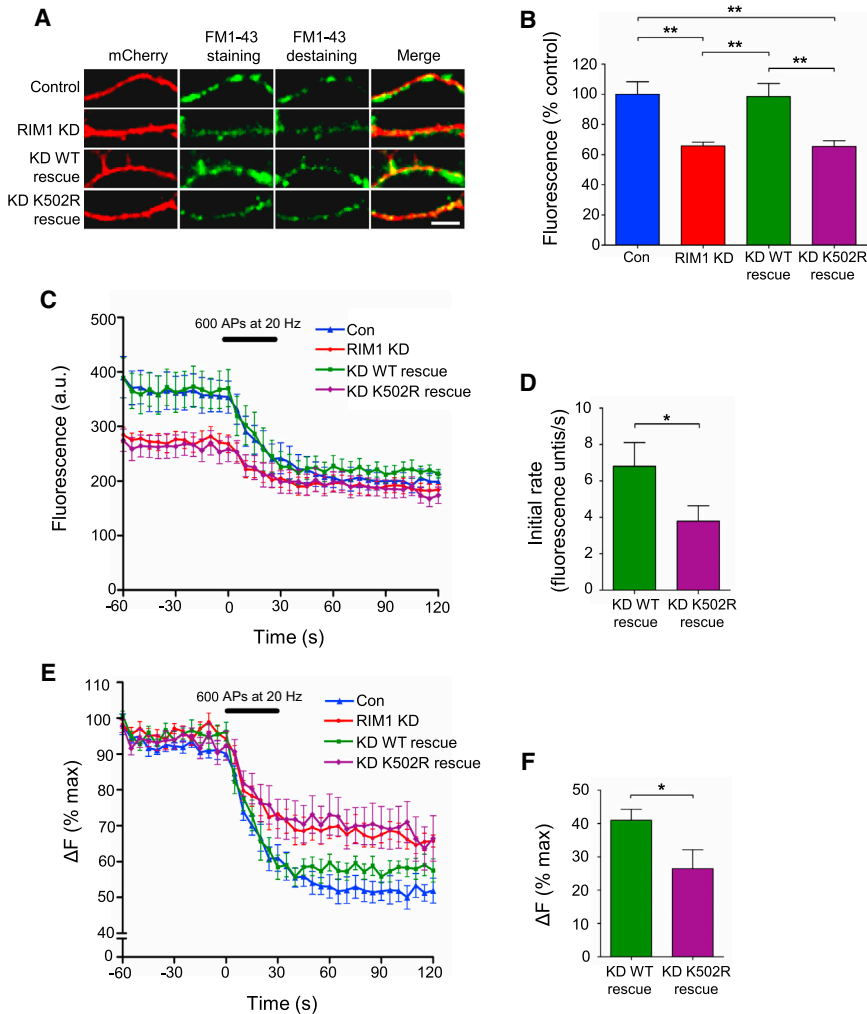


Figure 2. RIM1 α SUMOylation Is Involved in Synaptic Vesicle Cycling

(A) Representative images show FM1-43 uptake in hippocampal neurons treated with shRNA RIM1 KD and rescue with WT or nonSUMOylatable K502R RIM1 α . Destained images correspond to 30 s poststimulation. The scale bar represents 5 μ m.

(B) Quantification of (A) is presented. Normalized fluorescence is FM1-43 pixel intensity as percentage (%) of mean fluorescence of control (Con) neurons (n = 9–13). **p < 0.01 (one-way ANOVA). Data are represented as mean \pm SEM.

(C) Time course shows FM1-43 unloading triggered by 600 APs at 20 Hz for control, RIM1 KD, WT rescue, and K502R rescue neurons (n = 9–13), normalized to terminal background. Data are represented as mean \pm SEM.

(D) Quantification of the slope during the initial 15 s of stimulation in (C) is shown. *p < 0.05 (Student's t test). Data are represented as mean \pm SEM.

(E) Data in (C) are normalized to the average baseline (60 s prestimulus).

(F) Mean normalized FM1-43 release in (E) after 30 s of stimulation is shown. *p < 0.05 (Student's t test). Data are represented as mean \pm SEM. (% max), percent maximum.

See also Figure S2.

multidomain protein but contains only one lysine (K502) within a consensus SUMOylation motif (Figure 1E). We were able to SUMOylate RIM1 α in a HEK cell-based SUMO assay using SUMO-GG (in which the C-terminal diglycine conjugation motif has been exposed), but not SUMO- Δ GG (in which the conjugation motif has been deleted) (Figure 1F). Mutation of this lysine to arginine (K502R) or mutation of hydrophobic residue in the consensus site (A501S) completely prevented RIM1 α SUMOylation, confirming that K502 is the sole SUMO-1 attachment site (Figures 1G and S1A).

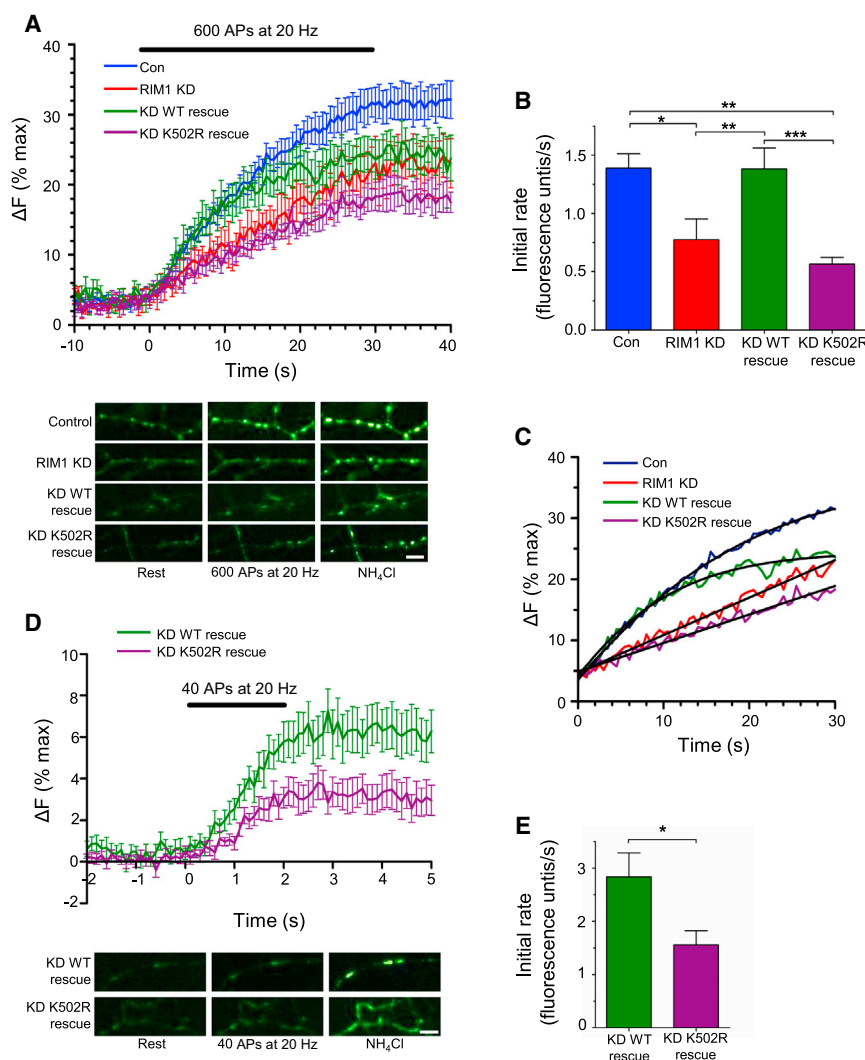
RIM1 α SUMOylation Regulates the Synaptic Vesicle Cycle

We used shRNA to knock down endogenous RIM1 and replaced it with nonSUMOylatable K502R RIM1 α . In HEK293T cells, there was a >90% knockdown (KD) of cotransfected RIM1 α , and this was effectively rescued by shRNA-insensitive constructs (“rescue”; Figures S2A and S2B). In hippocampal neurons, there was a ~65% KD of endogenous RIM (Figures S2C and S2D), with equivalent levels of replacement with WT or K502R RIM1 α that both displayed similar synaptic colocalization with synap-

sin-1 (Figures S2E and S2F). These results indicate that SUMOylation is not required for RIM1 α localization at the active zone.

To determine the roles of RIM1 α in pre-synaptic exocytosis, we used styryl FM dye loading (Gaffield and Betz, 2006). In RIM1 KD neurons, FM1-43 dye loading in response to depolarization was significantly reduced. Replacement with WT, but not K502R or A501S RIM1 α , rescued this defect (Figures 2A, 2B, S2G, and S2H), indicating that RIM1 removal slows or inhibits the synaptic vesicle cycle and that SUMOylation of the replacement RIM1 α is required to rescue this defect.

We next investigated exocytosis by measuring FM1-43 unloading in response to electrical field stimulation of neurons (Burrone et al., 2006), using 600 APs at 20 Hz, which induces exocytosis of the releasable synaptic vesicle pool (Fernández-Alfonso and Ryan, 2004). Neurons rescued with WT RIM1 α responded identically to control, non-shRNA-treated cells, whereas in contrast, K502R RIM1 α failed to rescue the KD phenotype (Figure 2C). Cells rescued with K502R RIM1 α displayed a significant reduction in the initial rate of FM1-43 unloading (taken as the initial 15 s during which all profiles were linear) compared to cells rescued with WT RIM1 α (Figures 2C and 2D). Additionally, the total amount of FM1-43 unloading was significantly lower in cells rescued with K502R RIM1 α (Figures 2E and 2F). Together, these data show that RIM1 α SUMOylation is necessary for normal stimulus-evoked synaptic vesicle exocytosis.



RIM1 α SUMOylation Has a Critical Role in the Fast Phase of Synaptic Vesicle Exocytosis, but Not Vesicle Docking or Priming

We further investigated the role of RIM1 α SUMOylation using the fluorescent indicator synaptophysin-pHluorin (SyphHy) to visualize vesicle fusion events (Burrone et al., 2006). Consistent with the FM1-43 experiments, RIM1 KD dramatically altered the kinetics of exocytosis of the synaptic vesicle pool (evoked by 600 APs at 20 Hz), and this was rescued by WT RIM1 α , but not K502R RIM1 α (Figures 3A–3C). The initial linear rate of exocytosis during the first 10 s of stimulation was reduced by ~50% in RIM1 KD and K502R RIM1 α -rescued neurons compared to control and WT RIM1 α -rescued neurons (Figures 3A–3C). Furthermore, as in the FM dye experiments, RIM1 KD neurons rescued with WT RIM1 α displayed similar rates of release to controls not treated with shRNA, which was different from the rates observed in RIM1 KD and K502R RIM1 α -rescued cells. However, although RIM1 α WT expression rescues the initial vesicle release rate, it does not completely recover the total level of exocytosis (Figures 3A–3C). We attribute this to the KD of

Figure 3. RIM1 α SUMOylation Has a Critical Role in Fast Synaptic Vesicle Exocytosis

(A) SyphHy fluorescence measurement of the releasable synaptic vesicle pool ($n = 6-9$) is shown. Fluorescence is normalized to baseline and expressed as percentage (%) of total SyphHy signal. Panels are representative images of SyphHy fluorescence taken at rest (0 s), after 600 APs at 20 Hz (30 s), and after NH₄Cl wash. Data are represented as mean \pm SEM. The scale bar represents 5 μ m. (B) Quantification of the initial rate of exocytosis in (A) is presented. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (one-way ANOVA). Data are represented as mean \pm SEM.

(C) Data from (A) are plotted to show the 30 s period of field stimulation (error bars removed for clarity). Release profiles of control and WT rescue cells are best described by an exponential function, whereas RIM1 KD and K502R rescue cells follow a linear profile. Data are represented as mean.

(D) Exocytosis from RRP ($n = 10$), imaged at 10 Hz, is shown. Fluorescence is normalized to the baseline and expressed as percent (%) total SyphHy signal (obtained with NH₄Cl). Panels below are representative images of SyphHy fluorescence. Data are represented as mean \pm SEM. The scale bar represents 5 μ m.

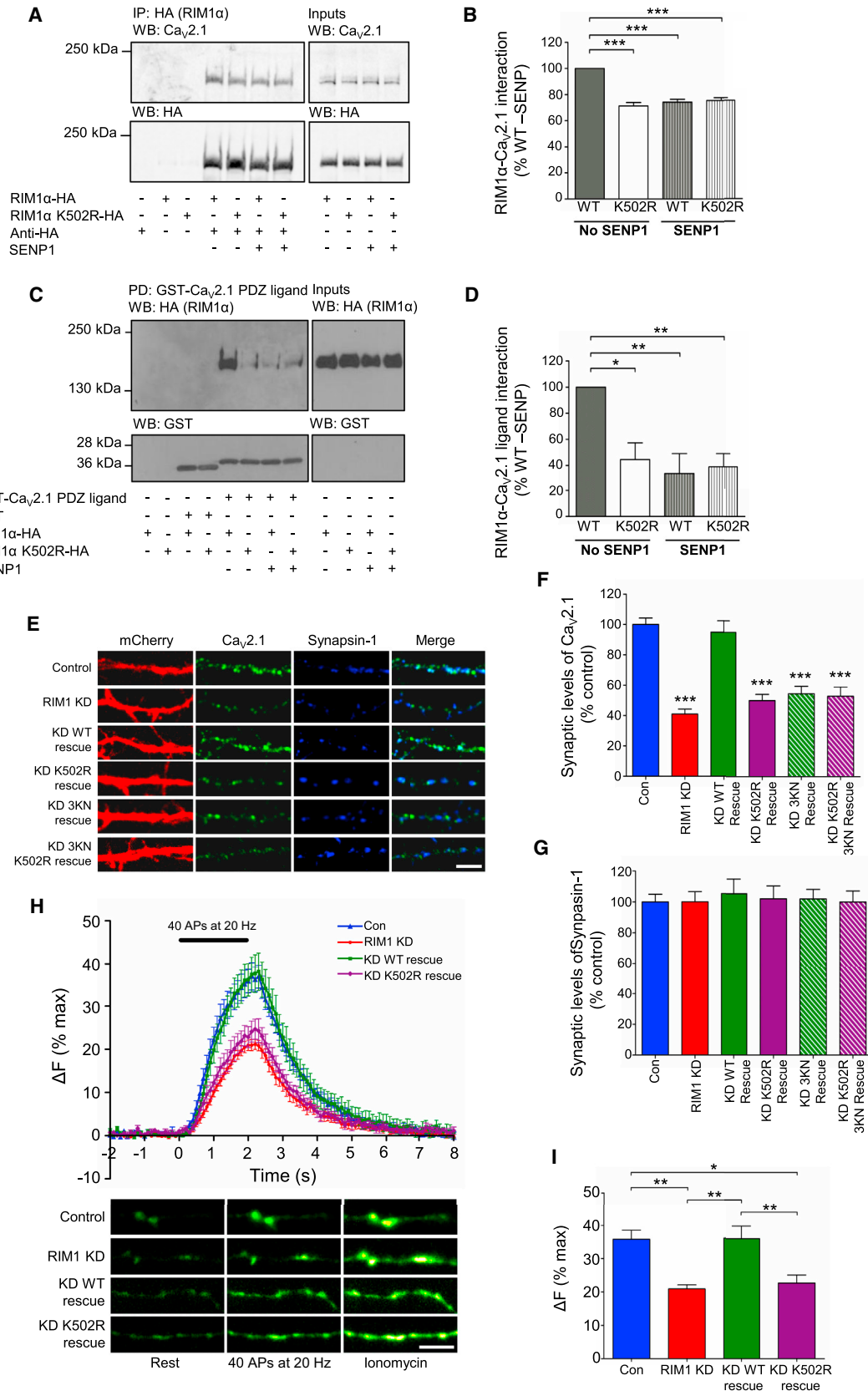
(E) Quantification of the rate of RRP release (relative slope during the 2 s of stimulation) is presented. * $p < 0.05$ (Student's *t* test). Data are represented as mean \pm SEM.

See also Figure S2.

both RIM1 α and RIM1 β but rescue with only the RIM1 α isoform. Thus, as previously reported by Kaeser et al. (2008), these results also implicate RIM1 β in regulation of synaptic strength. In these experiments, the presence of bafilomycin

A blocks vesicle reacidification, allowing specific measurement of exocytosis in the absence of endocytosis. Thus, we can state with confidence that the effects we see are specific for exocytosis, independent of any contribution of compensatory endocytosis.

Exocytosis from control and WT RIM1 α -rescued cells displays a biphasic release profile, whereas the RIM1 KD and K502R RIM1 α -rescued cells have a linear release profile (Figure 3C). To test whether this was due to the loss of the fast, initial phase of release from RIM1 KD and K502R-rescued neurons, we selectively measured primed synaptic vesicles ready to be released immediately on membrane depolarization (the readily releasable pool; RRP) using 40 APs at 20 Hz (de Jong et al., 2012) (Figure 3D). During the 2 s stimulation, there was an ~50% reduction in rate of exocytosis in cells rescued with K502R RIM1 α compared with WT RIM1 α -rescued cells (Figure 3E). These results show that exocytosis of the primed vesicles in the RRP is significantly impaired (resulting in a smaller apparent RRP) in the cells expressing K502R RIM1 α . This defect in exocytosis is unlikely to be due to a defect in docking or priming of



(legend on next page)

vesicles because this would result in a decrease in RRP size but the same rate of release. Thus, we conclude that RIM1 α SUMOylation has a critical role in fast phase of synaptic vesicle exocytosis.

RIM1 α SUMOylation Has a Critical Role in Depolarization-Evoked Presynaptic Ca²⁺ Entry due to a SUMO-Dependent PDZ Domain Interaction of RIM1 α with Ca_v2.1

The initial fast phase of synaptic vesicle exocytosis is called “synchronous release” because it occurs in direct response to Ca²⁺ entry through Ca²⁺ channel clusters at release sites (Gundelfinger and Fejtova, 2012). RIM1 α plays a major role in maintaining Ca²⁺ channel clusters via a direct PDZ interaction between RIM1 α and P/Q-type Ca²⁺ channels (Kaeser et al., 2011) and through an indirect complex formation involving RIM-binding protein (Liu et al., 2011). Intriguingly, the altered kinetics of exocytosis we see are similar to previous reports using P/Q-type Ca²⁺ channel inhibitors (Li et al., 2011). Following expression in cortical neurons, nonSUMOylatable K502R RIM1 α bound significantly less to Ca_v2.1 than WT RIM1 α . Consistently, deSUMOylation of neuronal lysate using SENP1 reduced WT RIM1 α binding to Ca_v2.1 to the same level as the K502R RIM1 α mutant (Figures 4A and 4B). These results indicate that RIM1 α SUMOylation directly regulates its binding to Ca_v2.1. Importantly, consistent with RIM1 α active zone localization (Schoch et al., 2002) and vesicle docking (Dulubova et al., 2005) being unaffected by SUMOylation, WT and K502R RIM1 α display equivalent binding to other active zone proteins, e.g., Liprin α 3 and Rab3 (Figures S3A and S3B). We found no evidence that Ca_v2.1 is a SUMO-binding protein, so our hypothesis is that SUMOylation enhances RIM1 α binding to Ca_v2.1. In agreement with this, K502R RIM1 α and SENP-treated WT RIM1 α bound the Ca_v2.1 PDZ ligand significantly less than WT RIM1 α (Figures 4C and 4D). This does not represent a general modification of the availability of the PDZ domain in RIM1 α by SUMOylation, given that the interaction between ELKS1b/2 and RIM1 α (also via the PDZ domain) was not affected by SUMOylation (Figures S3A and S3B). Furthermore, Ca_v2.1-clustering defects in RIM1 KD neurons were effectively rescued by the expression of WT RIM1 α , but not K502R RIM1 α or a RIM1 α

PDZ domain-deficient construct, in which three critical PDZ domain lysines (Kaeser et al., 2011) were mutated to asparagine (3KN), (Figures 4E, 4F, S3C, and S3D). Synapsin-1 showed no differences in clustering under any of the conditions (Figures 4E and 4G). Taken together, these data demonstrate that RIM1 α SUMOylation has a previously unreported role in PDZ interaction-mediated Ca_v2.1 clustering.

We next tested whether RIM1 α SUMOylation is required for normal presynaptic Ca²⁺ signaling, using a presynaptically targeted Ca²⁺-sensitive GFP reporter (SyGCaMP3) (Li et al., 2011). Ca²⁺ signals evoked by 40 APs at 20 Hz were significantly reduced (by ~40%) in RIM1 KD neurons, a defect that was rescued by expression of WT but not K502R RIM1 α (Figures 4H and 4I). These data strongly suggest that SUMOylated RIM1 α maintains normal presynaptic Ca²⁺ signaling via enhanced interactions with Ca_v2.1, which is required for synchronous synaptic vesicle exocytosis.

We note that our use of acute shRNA KD of RIM1 produced larger effects on activity-dependent Ca²⁺ entry and exocytosis than previous studies using knockout mice or genetic ablation in 3–5 days in vitro (DIV) neurons (e.g., Kaeser et al., 2012), in which deletion of both RIM1 α and RIM2 α was required to see such an effect. We perform our RIM1 KD in more mature neurons (10–11 DIV), in which active zone architecture is likely to be more established, therefore providing less scope for compensation for the loss of RIM1 by RIM2. Therefore, we believe that our approach gives a better indication of the role of the RIM1 isoform in mature synapses.

In this study, we show a previously unsuspected role for protein SUMOylation in the control of synchronous synaptic vesicle exocytosis. Specifically, we have shown a mechanism whereby SUMOylation causes the PDZ domain of RIM1 α to become available for interaction with Ca_v2.1. This is required to promote the clustering of Ca_v2.1 and Ca²⁺ entry on arrival of APs at the presynaptic terminus. Although we cannot formally exclude the possibility that these presynaptic effects are due to retrograde signaling from transfected postsynaptic neurons, given the internal consistency of our different functional approaches and their close correlation with the literature, we consider this explanation unlikely. We propose that SUMOylation can act as a molecular switch in the active zone, controlling the interactions and

Figure 4. RIM1 α SUMOylation Has a Critical Role in Synaptic Ca²⁺ Channel Clustering and Ca²⁺ Influx

(A) Representative blots show Ca_v2.1 interaction with WT and K502R RIM1 α in neurons.

(B) Quantification of (A) (n = 4) is shown. Data are presented as percent (%) WT interaction levels without SENP1. ***p < 0.001 (one-way ANOVA). Data are represented as mean \pm SEM.

(C) Representative blots show GST-Ca_v2.1-PDZ interaction with WT and K502R RIM1 α in neurons. Pull-downs with GST-Ca_v2.1-PDZ were blotted for HA (RIM1 α) and GST.

(D) Quantification of (C) (n = 5) is shown. Data are presented as percent (%) WT interaction levels without SENP1. *p < 0.05 and **p < 0.01 (one-way ANOVA). Data are represented as mean \pm SEM.

(E) Representative images show Ca_v2.1 levels (green) in synapsin-1-dense regions (blue) in RIM1 KD and rescue hippocampal neurons. The scale bar represents 5 μ m.

(F) Quantification of synaptic Ca_v2.1 in (E) (n = 12–43) is shown. ***p < 0.001 (one-way ANOVA) compared to both control and WT rescue. Data are represented as mean \pm SEM.

(G) Quantification of synapsin-1 in (E) is shown. Data are represented as mean \pm SEM.

(H) Presynaptic Ca²⁺ influx measured by SyGCaMP3 fluorescence (n = 8–10) is presented. SyGCaMP3 fluorescence is normalized to baseline and expressed as percent (%) maximum SyGCaMP3 signal obtained with 5 μ M ionomycin. Panels below are representative images of SyGCaMP3 fluorescence. Data are represented as mean \pm SEM. The scale bar represents 5 μ m.

(I) Quantification of SyGCaMP3 fluorescence after 2 s of stimulation is shown. *p < 0.05 and **p < 0.001 (one-way ANOVA). Data are represented as mean \pm SEM. See also Figures S2 and S3.

defining the function of different pools of multifunctional proteins, such as RIM1 α . Specifically in this case, SUMOylated RIM1 α is involved in the clustering of Ca²⁺ channels required for coordinated Ca²⁺ entry at the presynapse, whereas nonSUMOylated RIM1 α participates in functions such as vesicle priming and docking. Such a molecular switch would help to explain how several active zone proteins participate in many diverse functions. Defining exactly how this SUMO-dependent “switching” between RIM1 α -binding proteins orchestrates interactions at the active zone will provide important insight into synaptic function and dysfunction.

EXPERIMENTAL PROCEDURES

Molecular Biology

Cloning of all constructs was carried out with standard molecular biology techniques.

Biochemistry

Cultured rat cortical neurons (18 DIV) or HEK293T cells were used for SDS-PAGE, immunoblotting, immunoprecipitations, and GST pull-downs. Lysates were incubated \pm 20 mM NEM at 37°C for 30 min. HEK293T cell SUMOylation assays were conducted as described (Craig et al., 2012). For interactor studies, neurons infected with WT or K502R RIM1 α -HA Sindbis virus were lysed on ice \pm 20 nM SENP1, and interactions were probed using either anti-HA immunoprecipitation or a GST-tagged Ca_v2.1 PDZ ligand (sequence SEDDWC).

Neuronal Cultures and Imaging

Embryonic rat hippocampal and cortical neurons were prepared as described (Martin and Henley, 2004). Neurons were typically transfected at 11 DIV and imaged 4 days later (15 DIV). Immunocytochemistry assays were performed with paraformaldehyde fixation according to standard protocols. Image and blot analysis was performed using ImageJ software, and statistical analysis was conducted using GraphPad Prism. For functional fluorescence assays, one cell with 10–13 ROIs was analyzed per repeat. Fluorescence data were first normalized to baseline and then to maximal values (F_{max}).

Fluorescent Exocytosis Assays

For FM dye experiments, hippocampal neurons were transfected with RIM1 shRNA (mCherry) and either a WT or K502R RIM1 α -HA rescue construct. FM1-43 experiments were performed as described by Gaffield and Betz (2006) with 0.2 Hz imaging. SypHy and SyGCaMP3 were expressed on a pFIV RIM1 shRNA vector and cotransfected with WT or K502R mCherry-IRES-RIM1 α . SypHy experiments were performed as described by Burrone et al. (2006) with imaging at 0.5, 2, or 10 Hz. SyGCaMP3 experiments used 10 Hz imaging (Li et al., 2011). Electrical field stimulation was used for all assays: 600 APs at 20 Hz to induce exocytosis of the releasable synaptic vesicle pool, 40 APs at 20 Hz for RRP release, and SyGCaMP3 experiments.

Statistical Analysis

All quantified results shown are the mean \pm SEM. Statistical analyses were performed using either Microsoft Excel or GraphPad Prism. For comparison of two sets of data, one-tailed Student's *t* test was used. For comparison of multiple sets of data, one-way ANOVA with Bonferroni's post hoc test was used.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.10.039>.

AUTHOR CONTRIBUTIONS

F.G. performed all of the biochemistry, molecular biology, and immunocytochemistry and most of the live-imaging studies and assisted in preparing the manuscript. T.J.C. performed all of the SypHy studies, developed imaging protocols, directed the project, and assisted in preparing the manuscript. D.L.R. performed the initial Ubc9 interaction screen. J.M.H. oversaw the project and prepared the manuscript. All authors discussed the results and commented on the manuscript.

ACKNOWLEDGMENTS

We thank Kai Murk for the pFIV and mCherry-IRES vectors, Phil Rubin for virus preparation, and Jon Brown for technical assistance. We also thank Ruud Toonen, Kevin Wilkinson, Keri Hildick, and Nadia Jaafari for expert experimental advice. We gratefully acknowledge Alan Morgan for pCMV RIM1 α , Ruud Toonen for pCNA3 SypHy, and Susan Voglmaier for pCNA3 SyGCaMP3. This work was funded by the ERC, BBSRC, and MRC.

Received: July 23, 2013

Revised: October 1, 2013

Accepted: October 24, 2013

Published: November 27, 2013

REFERENCES

- Burrone, J., Li, Z., and Murthy, V.N. (2006). Studying vesicle cycling in presynaptic terminals using the genetically encoded probe synaptopHluorin. *Nat. Protoc.* 1, 2970–2978.
- Calakos, N., Schoch, S., Südhof, T.C., and Malenka, R.C. (2004). Multiple roles for the active zone protein RIM1alpha in late stages of neurotransmitter release. *Neuron* 42, 889–896.
- Castillo, P.E., Schoch, S., Schmitz, F., Südhof, T.C., and Malenka, R.C. (2002). RIM1alpha is required for presynaptic long-term potentiation. *Nature* 415, 327–330.
- Coppola, T., Magnin-Luthi, S., Perret-Menoud, V., Gattesco, S., Schiavo, G., and Regazzi, R. (2001). Direct interaction of the Rab3 effector RIM with Ca²⁺ channels, SNAP-25, and synaptotagmin. *J. Biol. Chem.* 276, 32756–32762.
- Craig, T.J., Jaafari, N., Petrovic, M.M., Jacobs, S.C., Rubin, P.P., Mellor, J.R., and Henley, J.M. (2012). Homeostatic synaptic scaling is regulated by protein SUMOylation. *J. Biol. Chem.* 287, 22781–22788.
- de Jong, A.P., Schmitz, S.K., Toonen, R.F., and Verhage, M. (2012). Dendritic position is a major determinant of presynaptic strength. *J. Cell Biol.* 197, 327–337.
- Deng, L., Kaeser, P.S., Xu, W., and Südhof, T.C. (2011). RIM proteins activate vesicle priming by reversing autoinhibitory homodimerization of Munc13. *Neuron* 69, 317–331.
- Dulubova, I., Lou, X., Lu, J., Huryeva, I., Alam, A., Schneggenburger, R., Südhof, T.C., and Rizo, J. (2005). A Munc13/RIM/Rab3 tripartite complex: from priming to plasticity? *EMBO J.* 24, 2839–2850.
- Feligioni, M., Nishimune, A., and Henley, J.M. (2009). Protein SUMOylation modulates calcium influx and glutamate release from presynaptic terminals. *Eur. J. Neurosci.* 29, 1348–1356.
- Fernández-Alfonso, T., and Ryan, T.A. (2004). The kinetics of synaptic vesicle pool depletion at CNS synaptic terminals. *Neuron* 41, 943–953.
- Flotho, A., and Melchior, F. (2013). Sumoylation: a regulatory protein modification in health and disease. *Annu. Rev. Biochem.* 82, 357–385.
- Gaffield, M.A., and Betz, W.J. (2006). Imaging synaptic vesicle exocytosis and endocytosis with FM dyes. *Nat. Protoc.* 1, 2916–2921.
- Gundelfinger, E.D., and Fejtova, A. (2012). Molecular organization and plasticity of the cytomatrix at the active zone. *Curr. Opin. Neurobiol.* 22, 423–430.
- Hay, R.T. (2005). SUMO: a history of modification. *Mol. Cell* 18, 1–12.

- Jaafari, N., Konopacki, F.A., Owen, T.F., Kantamneni, S., Rubin, P., Craig, T.J., Wilkinson, K.A., and Henley, J.M. (2013). SUMOylation is required for glycine-induced increases in AMPA receptor surface expression (ChemLTP) in hippocampal neurons. *PLoS ONE* 8, e52345.
- Kaesler, P.S., Kwon, H.B., Chiu, C.Q., Deng, L., Castillo, P.E., and Südhof, T.C. (2008). RIM1alpha and RIM1beta are synthesized from distinct promoters of the RIM1 gene to mediate differential but overlapping synaptic functions. *J. Neurosci.* 28, 13435–13447.
- Kaesler, P.S., Deng, L., Wang, Y., Dulubova, I., Liu, X., Rizo, J., and Südhof, T.C. (2011). RIM proteins tether Ca²⁺ channels to presynaptic active zones via a direct PDZ-domain interaction. *Cell* 144, 282–295.
- Kaesler, P.S., Deng, L., Fan, M., and Südhof, T.C. (2012). RIM genes differentially contribute to organizing presynaptic release sites. *Proc. Natl. Acad. Sci. USA* 109, 11830–11835.
- Koushika, S.P., Richmond, J.E., Hadwiger, G., Weimer, R.M., Jorgensen, E.M., and Nonet, M.L. (2001). A post-docking role for active zone protein Rim. *Nat. Neurosci.* 4, 997–1005.
- Li, H., Foss, S.M., Dobry, Y.L., Park, C.K., Hires, S.A., Shaner, N.C., Tsien, R.Y., Osborne, L.C., and Voglmaier, S.M. (2011). Concurrent imaging of synaptic vesicle recycling and calcium dynamics. *Front Mol Neurosci* 4, 34.
- Liu, K.S., Siebert, M., Mertel, S., Knoche, E., Wegener, S., Wichmann, C., Matkovic, T., Muhammad, K., Depner, H., Mettke, C., et al. (2011). RIM-binding protein, a central part of the active zone, is essential for neurotransmitter release. *Science* 334, 1565–1569.
- Lonart, G. (2002). RIM1: an edge for presynaptic plasticity. *Trends Neurosci.* 25, 329–332.
- Martin, S., and Henley, J.M. (2004). Activity-dependent endocytic sorting of kainate receptors to recycling or degradation pathways. *EMBO J.* 23, 4749–4759.
- Martin, S., Nishimune, A., Mellor, J.R., and Henley, J.M. (2007). SUMOylation regulates kainate-receptor-mediated synaptic transmission. *Nature* 447, 321–325.
- Müller, M., Liu, K.S., Sigrist, S.J., and Davis, G.W. (2012). RIM controls homeostatic plasticity through modulation of the readily-releasable vesicle pool. *J. Neurosci.* 32, 16574–16585.
- Schoch, S., Castillo, P.E., Jo, T., Mukherjee, K., Geppert, M., Wang, Y., Schmitz, F., Malenka, R.C., and Südhof, T.C. (2002). RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. *Nature* 415, 321–326.
- Schoch, S., Mittelstaedt, T., Kaesler, P.S., Padgett, D., Feldmann, N., Chevalleyre, V., Castillo, P.E., Hammer, R.E., Han, W., Schmitz, F., et al. (2006). Redundant functions of RIM1alpha and RIM2alpha in Ca²⁺-triggered neurotransmitter release. *EMBO J.* 25, 5852–5863.
- Shalizi, A., Gaudillière, B., Yuan, Z., Stegmüller, J., Shirogane, T., Ge, Q., Tan, Y., Schulman, B., Harper, J.W., and Bonni, A. (2006). A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. *Science* 311, 1012–1017.
- Südhof, T.C., and Rizo, J. (2011). Synaptic vesicle exocytosis. *Cold Spring Harb. Perspect. Biol.* 3, a005637.
- Wang, Y., Okamoto, M., Schmitz, F., Hofmann, K., and Südhof, T.C. (1997). Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. *Nature* 388, 593–598.