The Budding Yeast Amphiphysin Complex Is Required for Contractile Actin Ring (CAR) Assembly and Post-Contraction GEF-Independent Accumulation of Rho1-GTP

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Abstract

The late events of the budding yeast cell division cycle, cytokinesis and cell separation, require the assembly of a contractile actomyosin ring (CAR), primary and secondary septum formation followed by enzymatic degradation of the primary septum. Here we present evidence that demonstrates a role for the budding yeast amphiphysin complex, a heterodimer comprising Rvs167 and Rvs161, in CAR assembly and cell separation. The iqg1-1 allele is synthetically lethal with both rvs167 and rvs161 null mutations. We show that both iqg1 and the amphiphysin complex are required for CAR assembly in early anaphase but cells are able to complete assembly in late anaphase when these activities are, respectively, either compromised or absent. Absent amphiphysin dependent CAR assembly is dependent upon the Rvs167 SH3 domain, but this function is insufficient to explain the observed synthetic lethality. Dosage suppression of the iqg1-1 allele demonstrates that endocytosis is required for the default cell separation pathway in the absence of CAR contraction but is unlikely to be required to maintain viability. The amphiphysin complex is required for normal, post-mitotic, localization of Chs3 and the Rho1 GEF, Rom2, which are responsible for secondary septum deposition and the accumulation of GTP bound Rho1 at the bud neck. It is concluded that a failure of polarity establishment in the absence of CAR contraction and amphiphysin function leads to loss of viability as a result of the consequent cell separation defect.

Introduction

Cytokinesis is the final stage in the mitotic cell division process whereby cell separation is effected to generate two daughter cells. In yeast and animal cells this requires the assembly of a contractile actin ring (CAR) that initiates membrane ingression followed by enzymatic degradation of the primary septum. Here we present evidence that demonstrates a role for the budding yeast amphiphysin complex, a heterodimer comprising Rvs167 and Rvs161, in CAR assembly and cell separation. The iqg1-1 allele is synthetically lethal with both rvs167 and rvs161 null mutations. We show that both iqg1 and the amphiphysin complex are required for CAR assembly in early anaphase but cells are able to complete assembly in late anaphase when these activities are, respectively, either compromised or absent. Absent amphiphysin dependent CAR assembly is dependent upon the Rvs167 SH3 domain, but this function is insufficient to explain the observed synthetic lethality. Dosage suppression of the iqg1-1 allele demonstrates that endocytosis is required for the default cell separation pathway in the absence of CAR contraction but is unlikely to be required to maintain viability. The amphiphysin complex is required for normal, post-mitotic, localization of Chs3 and the Rho1 GEF, Rom2, which are responsible for secondary septum deposition and the accumulation of GTP bound Rho1 at the bud neck. It is concluded that a failure of polarity establishment in the absence of CAR contraction and amphiphysin function leads to loss of viability as a result of the consequent cell separation defect.

A second major role for actin in S. cerevisiae is in endocytosis and the establishment of polarized cell growth [27]. CAR assembly requires a re-organization of the actin cytoskeleton with a switch from a polarized growth and endocytosis associated pattern to a central regulator of CAR structure and function is Iqg1p, the single IQGAP homologue. Iqg1 is an essential gene and mutants fail to assemble a CAR [9,10]. A large number of proteins involved in CAR function and cell separation have been described [2] and of particular relevance here are Hof1, Cyk3 and Inn1 [11,12,13]. Various interactions between these proteins and dependency relationships determining the order of bud neck recruitment have been described [10,14,15,16,12,13,17,18,19,20,21,22]. CAR assembly is also dependent upon the Rho1 GTPase, which is recruited to the division site through interaction with the Tps1/ Rom2 guanine nucleotide exchange factors (GEFs) during mitosis. Subsequently Rho1-GTP accumulates at the bud neck via a second mechanism requiring GEF independent interaction between the polybasic C-terminus of Rho1 and the plasma membrane [23,24,25]. More recently activation of Rho1 has been shown to be required for secondary septum formation but not for CAR contraction or cleavage furrow ingestion [26].

A second major role for actin in S. cerevisiae is in endocytosis and the establishment of polarized cell growth [27]. CAR assembly requires a re-organization of the actin cytoskeleton with a switch from a polarized growth and endocytosis associated pattern to actin incorporation into the CAR at the bud neck [28,5]. To date
only one additional component of the endocytic pathway, Bsp1, has been implicated in CAR assembly although its function in either cytokinesis or endocytosis remains to be fully elucidated [29]. The requirement for membrane curvature, assembly of protein complexes at sites of membrane deformation and membrane scission are common to both endocytosis and cytokinesis. In the case of endocytosis this leads to vesicle formation and internalization at the plasma membrane and a vast body of work has led to the proposal of a convincing model for this process in yeast [30,31]. A central player in this model is the heteroeromic yeast amphiphysin complex, containing Rvs167 and Rvs161. These proteins belong to a family of BAR domain proteins, both members of the sub-family of N-BAR containing proteins. Extensive physical and genetic analysis has demonstrated that the BAR domain is able to bind to the plasma membrane structures and act to develop membrane curvature [32,33]. This property is thought to underpin the role of the yeast amphiphysin complex in endocytosis and by extension to be the key property and functional activity of all amphiphysins [33]. In addition to defects in endocytosis rs167 and rs161 mutations confer salt sensitivity and actin cytoskeleton polarity perturbation [34,35,36,37].

Several studies have demonstrated that genetic interactions between genes involved in CAR and cell separation functions are manifested by synthetic lethality, consistent with the interpretation of genetic relationships uncovered by synthetic lethality that has emerged from extensive systematic genetic analysis in yeast [38]. These analyses have also been employed productively to elucidate the cytokinetic and cell separation processes in S. cerevisiae [39,40,17,29,20]. Amphiphysins have previously been implicated in aspects of cytokinesis in both S. cerevisiae and S. pombe. In budding yeast high level expression of actin resulted in the observation of a ring like actin structure at the bud neck that was physically and genetically distinct from the CAR, the formation of which was Rvs167-dependent [41]. In S. pombe Hob3, the Rvs161 orthologue, was shown to localize to the CAR in an actin dependent manner and in the null mutant the CAR contraction rate was reduced [42]. We therefore undertook to investigate a potential role for the budding yeast amphiphysin complex in cytokinesis in more detail and using a combination of genetic and imaging experiments demonstrate roles for the amphiphysin complex in CAR assembly and in the GEF independent accumulation of GTP bound Rho1 at the bud neck following CAR contraction.

Results

Synthetic lethality between iqq1-1 and amphiphysin mutations

We initially screened a number of mutations in genes encoding proteins involved in the regulation and function of the actin cytoskeleton for genetic interaction with the hypomorphic, temperature sensitive iqq1-1 allele. Three independent analyses all identified null mutations in rs161A and rs167A as being synthetically lethal in combination with iqq1-1 at 26°C, the permissive temperature for the conditional allele. The results from standard genetic analysis of crosses between iqq1-1 and rs167A and rs161A are presented in Figs. 1A and B, respectively. In total 30 tetrads were analysed for iqq1-1×rs167A yielding 5 parental ditypes, 6 non-parental ditypes and 19 tetratypes, close to the expected 1:1:4 ratio. In all cases [31] where co-segregation of the mutated loci was inferred spores were inviable on non-selective media at 26°C as indicated (Figs. 1A). In the iqq1-1×rs161A cross dissection of 31 tetrads yielded 5 parental ditypes, 5 non-parental ditypes and 18 tetratypes and again in all cases of inferred co-segregation of the two mutations [20] cells were inviable (Fig. 1B). Control crosses to wild type for all three mutations demonstrated that none exhibited spore germination or viability problems (iggl-1 93% viability and inviability did not segregate with the iggl-1 allele; rs167A and rs161A 100% viability). Microscopic examination revealed that all inviable double mutants exhibited a chained phenotype and that lethality occurred after three or four attempts to complete cell division. In order to examine the terminal phenotype in more detail we created iqq1-1 and control strains in which the sole source of the relevant amphiphysin gene was a 13-myc epitope tagged copy integrated into the genome under control of the MET3 promoter, thus rendering amphiphysin gene expression methionine dependent. These strains were tested for their ability to restore normal amphiphysin function. Rvs167-13Myc rescued all phenotypes associated with an rs167 null mutation; synthetic lethality with iggl-1 on methionine deficient media, salt sensitivity and actin cytoskeleton polarity (Fig. S1A, B and C). In contrast the rs161-13Myc allele, despite being able to restore viability to iggl-1 rs161A cells (Fig. S1D), exhibited a degree of salt tolerance that was intermediate between wild type and rs161A levels (Fig. S1E). As a result all subsequent depletion experiments described below focused on Rvs167. Initial experiments examined the morphological effects of Rvs167-13mhc depletion in iggl-1 and control strains. The data show that, on depletion of the protein in iggl-1 rs167A cells, multiple budded, chained cell bodies with de-polarized actin accumulate over a 15 hr time course (Fig. 1C). The initial appearance of chained cells in the population at 7.5 hrs was coincident with loss of the protein as revealed by western blotting (Fig. 1C) and with the maxima of small to medium bud sized cells exhibiting depolarization of the actin cytoskeleton (Fig. S1C). It was concluded from these data that loss of amphiphysin function in a strain where Iggl1 function was compromised resulted in a failure of cytokinesis and cell separation.

Cell cycle dependent Rvs167 localization to the bud neck

If Rvs167 functions in cytokinesis/cell separation then it might be predicted that the protein localizes to the bud neck region and that potentially it interacts with known components of the cytokinetic and cell separation machinery. Using a fully functional Rvs167-GFP fusion expressed from the native locus the dynamics of Rvs167 distribution was examined. Representative images of Rvs167-GFP at different stages of the cell cycle are shown in Fig. 2A. The kymograph illustrates clear re-polarization of Rvs167-GFP patches to the bud neck in large budded cells forming either a transient ring like structure (Fig. 2A, arrows) or concentrating either side of division plane (Figure 2A, arrowheads). Next co-localization of Rvs167-GFP with Myo1-telTomato relative to ring contraction was examined. In the time series depicted in Fig. 2B foci of Rvs167-GFP can be observed at the bud neck early in mitosis as the spindle pole bodies separate across the bud neck (40 sec–7 min) and at later stages coincident with the initiation of ring contraction (21 min 40 sec onwards). Thereafter there is a strong polarization of the signal following completion of ring contraction and CAR disassembly (26 min–40 min) as the spindle pole bodies transit from the periphery to the centre of the mother and daughter cells following mitotic spindle breakdown. An additional timed image reconstruction series through the alternative plane and focused entirely at the CAR again show that there are two phases of Rvs167 localization to the bud neck, the first coincides with the period immediately prior to and coincident with CAR contraction. During this phase highly dynamic small foci of fluorescence are observed and can be seen to co-localize
with the CAR for relatively brief periods of time (Fig. 2C, arrows). The second phase of localization occurs immediately subsequent to the completion of CAR contraction, coincident with septum formation, and shows an increase in the number of Rvs167-GFP foci at the bud neck. To further refine the timing of Rvs167 recruitment to the bud neck region Rvs167-GFP localization was monitored in comparison with CAR contraction (Myo1-tdTomato) and spindle pole body movement (Spc29-tdTomato) and the data represented as merged kymographs (Fig. 2D). As indicated, the data demonstrate that Rvs167 accumulates at the bud neck over a very short interval immediately prior to the initiation of CAR contraction and remains polarized to both mother and daughter sides of the bud neck for a substantial period of time thereafter (Fig. 2D). These two phases of Rvs167 localization suggested the possibility that the amphiphysin complex functions in both the CAR formation/contraction and septum formation pathways.

Rvs167 interacts with known contractile ring and septation components

As stated above if Rvs167 is involved in CAR assembly and co-localizes with the CAR then it might be anticipated that it interacts with CAR components. However from the data presented above (Figs. 2A and 2B) any such interaction is likely to be very transient. Bimolecular fluorescence imaging is a useful technique for the live imaging of protein/protein interactions and one property of the approach is that the interaction between the two components of

![Figure 1. The null mutants rvs167Δ and rvs161Δ are synthetically lethal with the hypomorphic iqg1-1 allele. Eight representative tetrads depicting (A) rvs167Δ iqg1-1 synthetic lethality and (B) rvs161Δ iqg1-1 synthetic lethality. The individual genotypes of the viable spores and the inferred double mutants are indicated, P = parental ditype, N = non-parental ditype, T = tetrapype. C Repression of ectopic Rvs167-Myc expression recapitulates rvs167Δ iqg1-1 synthetic lethality and the associated cytokinesis defect. Indicated strains were grown at 26°C, the permissive temperature for the iqg1-1 allele, in media lacking (−MET) or containing (+MET) methionine and scored at the indicated time points for percentage of cells displaying multiple buds (n = 300 cells). This experiment was repeated twice but the data presented are representative and derived from a single experiment. Rvs167-13Myc protein levels were assayed by western blot following the addition of methionine and compared to a constitutive α-tubulin control. The two left hand control lanes in both panels represent wild type control levels of α-tubulin at the beginning and end of the time course. The micrographs depict representative examples of the actin and nuclear distribution in the RVS167-13MYC rvs167Δ iqg1-1 cells after 15 hours growth −/+ methionine.

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Figure 2. Cell cycle distribution of dynamic Rvs167-GFP in wild type cells. A representative single cell image series of Rvs167-GFP distribution at different stages of the cell cycle are shown. For each image series a single z-section that dissected the bud neck was imaged every 1 second for 2 minutes using 800 ms exposures. Images were deconvolved and are presented as stills or kymographs. Cell cycle stage was inferred from bud size and the protein distribution relative to bud size are summarized, DIC images correspond to last time point in the series. Arrows indicate transient Rvs167-GFP signal observed at the bud neck and arrowheads show polarization of the signal to either side of the bud neck in late mitosis.
CAR formation in mitosis is delayed in both iqg1-1 and rvs167 mutants

As both Iqg1 and Rvs167 are involved in regulating aspects of actin cytoskeletal function the formation of the CAR was examined. To address this question the Rvs167-13Myc depletion strains described above were engineered to express a Tpm2-GFP fusion expressed from the native locus. TPM2 encodes one of two S. cerevisiae tropomyosin orthologues and use of the GFP fusion enables live cell imaging of F-actin structures such as the CAR [24]. During anaphase B, the number of iqg1-1 cells exhibiting Tpm2-GFP fluorescence at the bud neck was reduced relative to wild type indicating that even when grown at the permissive temperature, the iqg1-1 allele is not fully functional. Importantly depletion of exogenous Rvs167-13Myc in cells lacking endogenous Rvs167 (rvs167Δ) caused severe impairment to CAR formation and this phenotype was exacerbated in the iqg1-1 rvs167Δ double mutant (Figure 4A). In contrast, in telophase cells (marked by spindle break down), both wild type and Rvs167 deleted cells shared similar numbers of CAR containing cells whilst in the iqg1-1 rvs167Δ double mutant CAR numbers increased post-anaphase only to the parental iqg1-1 level. Rvs167-13Myc depletion had little effect on CAR formation at this cell cycle stage (Fig. 4A). These data suggest that CAR formation is biphasic with both Iqg1 and Rvs167 functioning to promote CAR formation in early anaphase B. Moving away from the set of depletion strains described above, we also examined CAR formation in wild type, iqg1-1 and rvs167Δ cells. Again we observed a reduction in anaphase B CAR formation in both mutant strains relative to wild type and CAR assembly appeared to be restored in telophase cells. To examine the anaphase B defect in more detail anaphase B cells were further sub-divided on the basis of mitotic spindle length to cell length ratio. Spindle length in anaphase B cells was divided by the overall cell length, as determined by the boundaries of cytoplasmic Tpm2-GFP fluorescence. Broadly the data demonstrate the same trend as above, namely that CAR formation is compromised in iqg1-1 and rvs167Δ cells during early anaphase B and CAR formation is restored late in the mitotic cycle, once cells fall within a class in which the ratio of spindle length to overall cell length lies between 0.8 and 1.0 (Fig. 4B). It is important to note that in this experiment there is 2.65 fold increase, relative to wild type, in the number of rvs167Δ anaphase B cells that fall within this latter classification (0.8–1.0). This means that if one simply amalgamates early, middle and late anaphase B stages into one group, the severity of the CAR formation defect associated with rvs167Δ cells is somewhat hidden (Fig. 4B). These data are consistent with the interpretation that both Iqg1 and Rvs167 play a role in CAR assembly during anaphase B and that subsequently a second mechanism dependent upon factors other than Rvs167 either assembles or stabilizes the CAR in late anaphase B and post-anaphase.
Synthetic lethality is independent of CAR assembly defects

The question remained as to whether the failure of CAR assembly correlated to loss of cell viability in \( rs167A \) \( \text{iqg1-1} \) and \( rs161A \) \( \text{iqg1-1} \) cells. To address this question we created strains in which the SH3 domain of \( Rvs167 \) was deleted and replaced by insertion of GFP. Genetic analysis revealed that double mutants between \( \text{iqg1-1} \) and \( rs167A \text{SH3::GFP} \) were viable (Fig. 5A). In total 47 tetrads were analyzed and 47/48 double mutants were viable (98%) however they had a reduced growth rate relative to WT and single mutant progeny (Fig. 5A) coupled to a chained morphological phenotype as observed by microscopy examination (Fig. 5B). Subsequently cells were assessed for CAR formation and the results demonstrate that \( rs167A \text{SH3::GFP} \) cells exhibit only a very mild reduction in CAR formation compared to controls. However in combination with \( \text{iqg1-1} \) the \( rs167A \text{SH3::GFP} \) allele caused a severe reduction in CAR formation further compromising the reduced levels observed in the \( \text{iqg1-1} \) single mutant. These data implicate the SH3 domain of \( Rvs167 \) in CAR formation but imply that additional amphiphysin function is required to maintain viability in \( \text{iqg1-1} \) cells. Consistent with this interpretation was the observation that simultaneous deletion of the GPA and SH3 domains of \( Rvs167 \) further reduced spore viability of double mutants with \( \text{iqg1-1} \) (data not shown) and a \( Rvs167 \text{ BAR-GPA-SH3} \) domain deletion resulted in synthetic lethality (Fig. 1A).

CAR contraction is compromised in \( \text{iqg1-1} \) at the permissive temperature but retains normal contractile behaviour in the absence of \( Rvs167 \) function

Next the contractile function of the CAR was examined in the strains indicated using live cell imaging of a Myo1-GFP fusion to monitor contraction. Examination of the representative image time series and the kymographs constructed from those series demonstrate that CAR contraction goes to completion in wild type and \( Rvs167 \) depleted cells (Fig. 6A). However all strains carrying the \( \text{iqg1-1} \) allele are severely compromised for CAR contraction. The kymographs fail to taper to the point of complete contraction and Myo1-GFP marked CAR imaged in the alternative plane, observed through the bud neck, can be seen to disassemble without contracting (Fig. 6A). These observations were confirmed in asynchronously growing \( \text{iqg1-1} \) \( Rvs167 \text{ MYO1-GFP} \) cells where CAR contraction failed and rings disassembled in 61% cells (Fig. 6B). In those cells where potential contraction was observed it often appeared asymmetric which might indicate that apparent contraction was an observational artefact resulting from collapsed CARs.

Primary septum formation is coupled to CAR contraction and can be represented by the dynamics of Inn1 behaviour that follows the progress of membrane ingression and is required for Chs2 activation [44]. Inn1-GFP contraction/ingression was seen to occur similarly in wild type and \( rs167A \) cells (Fig. 6C). However in \( \text{iqg1-1} \) cells an increase in aberrant Inn-GFP movement was observed and in a small proportion of cells no contraction occurred. Depletion of \( Rvs167 \text{-13myc} \) in the \( \text{iqg1-1} \) \( Rvs167A \) background increased the number of cells in which Inn1 ingression was absent. These data indicate that \( Rvs167 \) has no role in CAR contraction but may play a role in co-ordinating the CAR and the proteins required for primary septum formation. The data further implicate \( \text{iqg1} \) in contraction as well as assembly of the CAR.

Endocytosis required for CAR independent suppression of \( \text{iqg1-1} \) at the restrictive temperature but not for \( \text{iqg1-1} \) viability

The fact that CAR assembly and contraction defects failed to explain the observed synthetic lethality between \( \text{iqg1-1} \) and amphiphysin null mutations lead us to investigate other known aspects of amphiphysin function. Previous work has identified two dosage suppressors of the \( \text{iqg1-1} \) allele that define two distinct suppression pathways. High dosage expression of \( \text{Mlc}1 \) restores CAR formation to \( \text{iqg1-1} \) cells at the restrictive temperature whereas increased expression of \( \text{Bsp1} \) leads to CAR independent cell separation through a default pathway requiring extensive but disorganized deposition of septal/cell wall components [16,17]. The data presented in Fig. 7A demonstrate that increased \( \text{Mlc}1 \) expression is able to restore substantive growth to \( \text{iqg1-1} \) cells in the absence of \( Rvs167 \) expression. In contrast increased \( \text{Bsp1} \) expression fails to restore viability to \( \text{iqg1-1} \) cells lacking \( Rvs167 \) expression (Fig. 7B). One possibility that emerges from these data is that it is the endocytic function of \( Rvs167 \) that is required both for viability of \( \text{iqg1-1} \) cells and for \( \text{Bsp1} \) mediated suppression. Loss of endocytosis function is unlikely to explain the observed synthetic lethality as double mutants between \( \text{iqg1-1} \) and \( \text{abp1} \text{A} \) retain viability (Fig. 7C) as do \( \text{iqg1-1} \) \( \text{shl1} \text{A} \) and \( \text{iqg1-1} \) \( \text{cmn1} \text{A} \) cells (unpublished data). However CAR independent \( \text{iqg1-1} \) suppression does require efficient endocytosis as increased \( \text{Bsp1} \) expression fails to suppress loss of \( \text{iqg1-1} \) function in \( \text{iqg1-1} \) \( \text{abp1} \text{A} \) cells at the restrictive temperature (Fig. 7C). The failure of \( \text{Bsp1} \) mediated dosage suppression of \( \text{iqg1-1} \) in the absence of \( Rvs167 \text{Rvs161} \) does not result from altered localization of \( \text{Bsp1} \) to the CAR (Fig. 7D). Actin patch localization of \( \text{Bsp1} \text{GFP} \) exhibits the polarity defect previously associated with amphiphysin mutations [34].

Previous data demonstrated that \( \text{Chs3} \) is required for \( \text{Bsp1} \) dose dependent suppression of \( \text{iqg1-1} \) [29] suggesting the possibility that \( Rvs167 \) might regulate \( \text{Chs3} \) localization/activity. We therefore examined \( \text{Chs3} \text{GFP} \) localization in the absence of amphiphysin function. It had previously been shown that endocytic mutants exhibit increased levels of chitin synthase activity reflected in an accumulation of \( \text{Chs3} \) throughout the plasma membrane as opposed to a polar distribution within the bud and a loss of chitosomes, both phenotypes resulting from a defect in recycling of \( \text{Chs3} \) at the plasma membrane [46]. \( \text{Chs3} \) localization in \( \text{rs167A} \) \( \text{rs161A} \) cells is consistent with those earlier reports i. e. the absence of chitosomes and altered plasma membrane distribution (Fig. 7E). \( \text{Chs3} \) still accumulated at the plasma membrane
surrounding the bud neck and marked closed septa in large budded cells but the level of the protein at the bud neck appeared to be markedly reduced in amphiphysin mutants compared to control cells (Fig. 7E).

A further possibility is that machinery required for primary septum formation is altered in amphiphysin deficient cells, although it should be noted that neither rvs167Δ nor rvs161Δ cells exhibit known septation defects. We were unable to test Chs2 localization as epitope tagged alleles of this gene are inviable in our

Figure 4. The timing of actin ring assembly is delayed in iqg1-1 and rvs167Δ mutants. A indicated strains were grown to exponential phase in minimal media lacking methionine (−MET) before shifting to media containing methionine (+MET). Live cell images were acquired and the presence of Tpm2-GFP at the bud neck scored at the indicated time points. Mitotic progression stage was assessed by observation of mitotic spindle length using CFP-Tub3. Between 160–344 cells were scored from at each time point. The percent values derive from a single representative experiment. B Tpm2-GFP presence at the bud neck in wild type and single mutant parental strains. Cells were grown to exponential phase and live images captured and scored for Tpm2-GFP bud neck localization and anaphase B/post anaphase microtubule distribution (N values are indicated). C the same image set scored for Tpm2-GFP bud neck localization relative to mitotic progression. To quantify mitotic progression the ratio of anaphase spindle length to overall cell length was calculated and binned as indicated (N values as shown). The N values used in B and C derive from two independent repeats and the number of cells scored in each category in each experiment are indicated below the graphs.

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strain background. We did examine the behaviour of functional Hof1-GFP and Cyk3-GFP, thought to be required for Chs2 activation [44] in rvs167Δ cells and found both proteins to undergo similar dynamic localization patterns as observed in wild-type cells (Fig. S2). One important implication of this result is that there is no obvious affect on general bud neck structure in the absence of Rvs167, consistent with normal progress of Inn1-GFP ingression, which tracks primary septum formation (Fig. 6C).

Rom2 distribution is altered in rvs167Δ mutant

The altered distribution of Chs3 suggested the possibility that post-mitotic polarity establishment associated with secondary septum deposition and cell wall biosynthesis might be altered in rvs167Δ cells. The one other phenotype associated with a lack of amphiphysin function is the perturbation of actin cytoskeletal polarity [34]. The data presented in Fig. 8 demonstrate that dynamic behaviour of Rom2, one of two GEFs for Rho1, is altered in cells lacking amphiphysin function. In wild type and iqg1-1 cells Rom2-GFP accumulates at the bud neck in late mitosis and then distributes evenly to either side of the bud neck in parallel with secondary septum formation (Fig. 8A and B). In the absence of Rvs167 function Rom2 fails to accumulate evenly on either side of the bud neck (Fig. 8C). The second known Rho1-GEF, Tus1, behaves normally in all three strains (Fig. S3).

Rvs167 is required for Rho1-GTP localization to the bud neck

If normal Rom2 distribution is required for Rho1 activation at the point of cell separation then absence of Rvs167 might perturb Rho1 activity. Rho1 is known to function in polarity establishment during cytokinesis and cell separation and is required for secondary septum formation [25,26]. Rho1 undergoes two phases of localization, one GEF dependent requiring GDP to GTP bound Rho1 cycling during late mitosis and the second GEF independent accumulation of the GTP bound form requiring the poly-basic Rho1 C-terminus and likely interaction with PIP2 [25]. Using GFP-Rho1 and the GFP-Rho1-Q68H mutant [25], a GTP-locked form of Rho1, we examined the GEF independent localization of Rho1 in the presence and absence of Rvs167. Here we observed a strongly altered distribution pattern with a loss of polarization of GFP-Rho1-Q68H to the bud neck in telophase cells and an even distribution around the plasma membrane of both mother cell and buds in sharp contrast to wild type cells (Fig. 8).

Discussion

Amphiphysins are a conserved family of eukaryotic proteins involved in clathrin mediated endocytosis [33]. The budding yeast amphiphysin complex is a heterodimer formed between Rvs161 and Rvs167 with additional functions in salt tolerance and actin cytoskeletal polarity establishment [45]. Genetic analysis has demonstrated that rvs161 and rvs167 null mutations exhibit identical interactions which coupled to characterization of heterodimer formation and protein-protein interactions has led to the conclusion that the two proteins function as an obligate heterodimer [47]. Previous work has identified at least one phenotypic difference between the two mutants [48]. However,

Figure 5. The SH3 domain of Rvs167 is required for actin ring assembly. A representative tetrad analysis of the relevant heterozygous diploid strains and the relevant segregation pattern of the rvs167Δ::GFP and iqg1-1 alleles. B the indicated strains were grown to exponential phase in YEPD at permissive 26°C before fixation (3.7% formaldehyde) and DIC image acquisition. Scale bars = 2.5 μm. C formaldehyde fixed cells from above were stained with tritic-phalloidin (actin) and DAPI (DNA). WT (n = 295), 167 Δ (n = 304), RVS167-GFP (n = 290), RVS167::GFP (n = 344), iqg1-1 (n = 243), iqg1-1 RVS167-GFP (n = 452), iqg1-1 RVS167::GFP (n = 222). The n values derive from a single representative experiment.
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apart from the distinct BiFC interaction between Rvs161 and Iqg1 (Fig. 3C) that we interpret to reflect a genuine interaction of the Rvs161/Rvs167 complex, we have never observed a phenotypic difference between the rvs161 and rvs167 null mutations in the course of these experiments. Here we have shown that rvs161 and rvs167 null mutants exhibit synthetic lethality with the hypomorphic iqg1-1 allele (Fig. 1). Iqg1, the sole S. cerevisiae IQGAP homologue, functions during cytokinesis and cell separation, it is a

Figure 6. Actomyosin contractile ring and Inn1-GFP dynamics are altered in iqg1-1 and iqg1-1 rvs167D mutants. A the indicated strains were grown to exponential phase in media lacking methionine and then imaged (−MET). Exponential −MET cells were transferred to media containing methionine and grown for 7.5 h to deplete Rvs167-13myc before imaging (+MET). Images were acquired every 35 seconds for 50 minutes (21z-sections, 0.2 μm spacing). Kymograph scale bar = 3.5 mins. B CAR contraction fails in iqg1-1 cells. Images acquired every 45 seconds for 45 minutes (21z-sections, 0.2 μm spacing). Kymograph scale bar = 6 mins. C the indicated strains were grown and imaged as described above. Kymograph scale bar = 3.5 mins. Cell numbers scored for symmetric, asymmetric or no contraction were: WT (n = 138), rvs167D (n = 145), iqg1-1 (n = 113), rvs167D iqg1-1 (n = 274). The n values in A and C derive from a single representative experiment.
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component of the CAR and is required for actomyosin ring assembly. Synthetic lethality between null and hypomorphic mutations implies that the proteins function in the same pathway [38] and we therefore conclude that the Rvs161/Rvs167 amphiphysin complex functions in cytokinesis and cell separation. Consistent with this interpretation depletion of either Rvs161 or Rvs167 in an iqg1-1 strain resulted in the appearance of chained cells (Fig. 1C). Analysis of the temporal and spatial pattern of an Rvs167-GFP fusion protein reveals transitory interactions with CAR reflected by co-localization with Myo1 (Fig. 2C). These interactions are observed prior to the initiation of CAR contraction (Fig. 2C and D). As contraction occurs Rvs167-GFP begins to accumulate further at the bud neck and persists beyond the point of CAR disassembly and completion of the cytokinetic phase of cell division, (Fig. 2A, B and D), behaviour that parallels the well characterized re-polarization of the actin cytoskeleton at this stage of the cell cycle.

On the basis of the results discussed above, that Iqg1 and the amphiphysins function in the same pathway(s) and Rvs167 transiently associates with the CAR a potential role for Rvs167 in CAR assembly was examined. In wild type a significant proportion (50%) of anaphase B cells have completed the assembly of the CAR (Fig. 7). MLC1 overexpression, but not BSP1, suppresses rvs167Δ iqg1-1 synthetic lethality. Indicated strains were grown to exponential phase in glucose containing media, washed into galactose/raffinose media and then spotted onto indicated media and grown at temperature shown before imaging. MLC1 overexpression suppresses rvs167Δ iqg1-1 synthetic lethality (A) whereas BSP1 overexpression does not (B). Endocytosis is required for BSP1-mediated suppression of iqg1-1 at the restrictive temperature. D BSP1 localisation unaltered in amphiphysin null cells. BS1-GFP and eCFP-TUB3 represent maximum and average intensity projections respectively. 5 representative examples of BSP1-GFP rings at the bud neck in the z/x plane (i.e. looking through the division site) are shown. Scale bar = 2.5 μm. (E) Chs3-GFP polarity altered in rvs mutant cells. Fluorescent images are maximum intensity projections. Image reconstructions of bud neck demonstrate Chs3-GFP localizes to rings and septa. Scale bar = 2.5 μm.

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Figure 8. Rom2-GFP fails to repolarise at the division site prior to cell separation in rvs167Δ cells. WT (A), iqg1-1 (B) and rvs167Δ (C) cells expressing Rom2-GFP were imaged every minute (3 second exposures, 18 z-sections, 0.2 μm z-spacing) for 45 minutes. Maximum intensity projections of deconvolved z-stacks are shown for indicated time points. Scale bars = 2 μm. (D) Percentage of WT (n = 54), iqg1-1 (n = 44) and rvs167Δ (n = 42) cells that repolarized Rom2-GFP to either side of the bud neck prior to cell separation was quantified for each strain. The n values derive from a single representative experiment.

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Figure 9. Rvs167 is required to localise active Rho1-GTP to the division site. A localisation of a wild-type GFP-Rho1 construct to the bud neck in telophase cells was largely unaltered in *iqg1-1* and *rvs167* mutants as shown in the fluorescent average intensity project images. Scale bar = 2.5 μm. B Quantitation of GFP-Rho1 localisation to the division site in telophase cells (*n* = 2). In total 169, 163 and 211 telophase WT, *iqg1-1* and *rvs167* cells were scored between two independent experiments and the scale bars represent standard deviation. C GTP-locked GFP-Rho1Q68H mutant fails to repolarize from bud tip to bud neck upon spindle breakdown in *rvs167* mutants, instead it often exhibited depolarized plasma.
membrane localisation (asterisk). D quantitation of GFP-Rho1<sup>Q68L</sup> telophase localization (n = 2). In total 289, 301 and 221 telophase WT, <i>iqg1-1</i> and <i>rvs167</i>D cells were scored between two independent experiments. Error bars in B and D are +/- standard deviation of the mean for repeat experiments. E summary of amphiphysin function during cytokinesis and cell separation. Anaphase B: <i>rvs167</i>161 (indicated in blue) and <i>iqg1</i> localise to the division site promoting CAR formation (purple circles). The SH3 domain of <i>rvs167</i> is required for this process. Spindle disassembly marks the start of telophase. Shortly thereafter CAR contraction is initiated driving centripetal invagination of the plasma membrane behind which the primary septum is formed (green bar). Concomitantly, <i>rvs167</i>161 patches (blue) concentrate at the division site triggering polarity establishment (red arrows). This is dependent upon the amphiphysin BAR domains that manipulate membrane curvature and microdomain lipid composition. Polarity establishment then drives assembly of septum formation specific protein complexes. <i>rvs167</i> is required for the polarised accumulation of active Rho1-GTP and its effector Chs3 at the bud neck and also mediates correct localisation of the Rho1 GEF, Rom2, during secondary septum formation (yellow bar). These and likely additional factors promote septum formation. <i>rvs167</i>161 remains associated at the division site following cell separation.

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of the CAR. Depletion of <i>rvs167</i> reduces this level and <i>iqg1-1</i> cells also exhibit a reduction in the number of CAR containing anaphase B cells. This defect is exacerbated in double <i>rvs167</i>A <i>iqg1-1</i> mutants (Fig. 4A). However in post-anaphase cells CAR numbers are reduced in <i>iqg1-1</i> cells but <i>rvs167</i> depleted cells attain similar levels to wild-type cells (Fig. 4A). Further analysis demonstrated that in <i>rvs167</i>A and <i>iqg1-1</i> mutants CAR assembly is compromised during the early stages of anaphase but that CAR can be able to recover this defect and assemble wild type (<i>rvs167</i>A) or near wild-type (<i>iqg1-1</i>) CAR numbers. These data imply that an additional factor can license CAR assembly in late mitosis and one candidate for this is Hof1p as a "null and BAR domain construct were synthetically lethal (<i>iqg1-1</i>) or near lethal (<i>rvs167</i>D) mutants CAR assembly is severely compromised and the CAR simply disassembled, when endocytosis is compromised [52]. The data presented here demonstrate that efficient endocytosis was unlikely to be required for viability but was necessary for CAR independent, Bsp1 mediated, suppression of the <i>iqg1-1</i> allele. This requirement presumably reflects the loss of vesicle recycling in the absence of endocytosis as evidenced by altered Chs3 distribution.

Amphiphysin Function in Cytokinesis

Secondary septum deposition and cell wall synthesis requires polarised assembly and activation of the relevant enzyme activities requiring catalytic components, Chs3 and Fks1 respectively. Both are effectors of activated, GTP bound, Rhol, which is localized to the plasma membrane at the bud neck through interaction between the poly-Basic Rhol C-terminus and PIP2 [25]. Loss of
| Strain number | Genotype | Source |
|---------------|----------|--------|
| SSC1          | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 | Laboratory collection |
| SSC2          | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 | Laboratory collection |
| SSC3          | MATa/s ade2-1/ade2-1 his3-11,15 his3-11,15 leu2-3,112/leu2-3,112 ura3-1/ura3-1 trp1-1/trp1-1 can1-100/can1-100 | Laboratory collection |
| SSC166        | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 iog1-1 | Laboratory collection |
| SSC619        | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 vs167.1::TRP1 | K. Ayscough |
| SSC1001       | MATa/s ade2-1/ade2-1 his3-11,15 his3-11,15 leu2-3,112/leu2-3,112 ura3-1/ura3-1 trp1-1/trp1-1 can1-100/can1-100 iog1-1/1/IQG1 vs167.1::TRP1/RVS167 | This study |
| SSC1614       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167-GFP::natMX6 | This study |
| SSC1636       | MATa/s ade2-1/ade2-1 his3-11,15 his3-11,15 leu2-3,112/leu2-3,112 ura3-1/ura3-1 trp1-1/trp1-1 can1-100/can1-100 iog1-1/1/IQG1 vs161.1::kanMX4/RVS161 | This study |
| SSC1659       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167-GFP::natMX6 iog1-1 | This study |
| SSC1754       | MATa ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::Yplac128-pMET3-RVS167-13MYC::LEU2 | This study |
| SSC1767       | MATa ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::Yplac128-pMET3-RVS167-13MYC::LEU2 | This study |
| SSC1792       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 IQG1-VC155::kanMX6 RVS167-VN173::His3MX6 | This study |
| SSC1794       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167-VC155::kanMX6 B5F1-VN173::His3MX6 | This study |
| SSC1853       | MATa ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::Yplac128-pMET3-RVS167-13MYC::LEU2 MYO1-GFP::natMX6 vs167.1::TRP1 | This study |
| SSC1854       | MATa ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::Yplac128-pMET3-RVS167-13MYC::LEU2 MYO1-GFP::natMX6 iog1-1 | This study |
| SSC1855       | MATa ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::Yplac128-pMET3-RVS167-13MYC::LEU2 MYO1-GFP::natMX6 vs167.1::TRP1 iog1-1 | This study |
| SSC1859       | MATa ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::Yplac128-pMET3-RVS167-13MYC::LEU2 MYO1-GFP::natMX6 | This study |
| SSC1987       | MATa/s ade2-1/ade2-1 his3-11,15 his3-11,15 leu2-3,112/leu2-3,112 ura3-1/ura3-1 trp1-1/trp1-1 can1-100/can1-100 RVS167-VC155::kanMX6/RVS167-VN173::His3MX6 | Laboratory collection |
| SSC2070       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167-VC155::kanMX6 CYK3-VN173::His3MX6 | This study |
| SSC2072       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167-VC155::kanMX6 HOF1-VN173::His3MX6 | This study |
| SSC2256       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167-VC155::kanMX6 CYK3-VN173::His3MX6 bsp1::::His3MX6 | This study |
| SSC2258       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167-VC155::kanMX6 CYK3-VN173::His3MX6 hof1::::kanMX6 | This study |
| SSC2293       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS161-VC155::kanMX6 IQG1-VN173::His3MX6 | This study |
| SSC2296       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS161-VC155::kanMX6 RVS167-VN173::His3MX6 | This study |
| SSC2299       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS161-VC155::kanMX6 HOF1-VN173::His3MX6 | This study |
| SSC2302       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS161-VC155::kanMX6 CYK3-VN173::His3MX6 | This study |
| SSC2305       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS161-VC155::kanMX6 B5F1-VN173::His3MX6 | This study |
| SSC2315       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167-VC155::kanMX6 CYK3-VN173::His3MX6 | This study |
| SSC2698       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 TPM2-GFP::natMX6 [YCplac33-pMET3-eCFP-TUB3::URA3] | This study |
| SSC2699       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 TPM2-GFP::natMX6 [YCplac33-pMET3-eCFP-TUB3::URA3] vs167.1::TRP1 | This study |
| SSC2700       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 TPM2-GFP::natMX6 [YCplac33-pMET3-eCFP-TUB3::URA3] iog1-1 | This study |
| SSC2724       | MATa ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::Yplac128-pMET3-RVS167-13MYC::LEU2 INN1-GFP::natMX6 | This study |
| SSC2725       | MATa ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::Yplac128-pMET3-RVS167-13MYC::LEU2 INN1-GFP::natMX6 vs167.1::TRP1 | This study |
| SSC2727       | MATa ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::Yplac128-pMET3-RVS167-13MYC::LEU2 INN1-GFP::natMX6 iog1-1 | This study |
| Strain number | Genotype                                                                 | Source               |
|---------------|---------------------------------------------------------------------------|----------------------|
| SSC2729       | MATa ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 INN1-1::GFP::natMX6 rvs167::TRP1 | This study           |
| SSC2735       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 GAL-CDC20::TRP1 cdc20::LEU2 RVS167-GFP::natMX6 MYO1+tdTomato::kanMX6 SPC29-tdTomato::hphMX6 | This study           |
| SSC2829       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167-VC155::kanMX6 INN1-1::His3MX6 | This study           |
| SSC2832       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS161-VC155::kanMX6 INN1-1::VNN173::Hls3MX6 | This study           |
| SSC2876       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167::GFP::natMX6 | This study           |
| SSC2880       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 RVS167::GFP::natMX6 | This study           |
| SSC2942       | MATa ade2-1 his3-11,15 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 ura3-1::YIplac211-pGAL1-eCFP-TUB::URA3 | This study           |
| SSC2944       | MATa ade2-1 his3-11,15 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 ura3-1::YIplac211-pGAL1-eCFP-TUB::URA3 TPM2-GFP::natMX6 | This study           |
| SSC2946       | MATa ade2-1 his3-11,15 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 ura3-1::YIplac211-pGAL1-eCFP-TUB::URA3 | This study           |
| SSC2948       | MATa ade2-1 his3-11,15 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 ura3-1::YIplac211-pGAL1-eCFP-TUB::URA3 | This study           |
| SSC2759       | MAT a ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 [pYEURA3-GAL1,10-MCL1::URA3] | This study           |
| SSC2760       | MAT a ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 [pYEURA3-GAL1,10-MCL1::URA3] {rvs167-t::TRP1} | This study           |
| SSC2761       | MAT a ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 [pYEURA3-GAL1,10-MCL1::URA3] {rvs167-t::TRP1} | This study           |
| SSC2762       | MAT a ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 [pYEURA3-GAL1,10-MCL1::URA3] {rvs167-t::TRP1} | This study           |
| SSC2090       | MAT a ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 [pYES2.0-GAL1-SPS1::URA3] | This study           |
| SSC2091       | MAT a ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 [pYES2.0-GAL1-SPS1::URA3] {rvs167-t::TRP1} | This study           |
| SSC2092       | MAT a ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 [pYES2.0-GAL1-SPS1::URA3] {ig1-t::TRP1} | This study           |
| SSC2093       | MAT a ade2-1 his3-11,15 ura3-1 trp1-1 can1-100 leu2-3,112::YIplac128-pMET3-RVS167-13MYC::LEU2 [pYES2.0-GAL1-SPS1::URA3] {ig1-t::TRP1} | This study           |
| SSC2743       | MATs ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 [YEplac181-pMET3-SPS1::LEU2] | This study           |
| SSC2744       | MATs ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 [YEplac181-pMET3-SPS1::LEU2] abp1-1::URA3 | This study           |
| SSC2745       | MATs ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 [YEplac181-pMET3-SPS1::LEU2] abp1-1::URA3 ig1-1 | This study           |
| SSC2746       | MATs ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 [YEplac181-pMET3-SPS1::LEU2] abp1-1::URA3 ig1-1 | This study           |
| SSC2183       | MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100 ura3-1::YIplac211-pGAL1-eCFP-TUB::URA3 BSP1::GFP::natMX6 | This study           |
| SSC2186       | MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100 ura3-1::YIplac211-pGAL1-eCFP-TUB::URA3 BSP1::GFP::natMX6 rvs167-t::HphMX6 | This study           |
| SSC1016       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 CH53-GFP::(S65T)::KanMX6 | This study           |
| SSC2055       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 CH53-GFP::(S65T)::KanMX6 rvs167-t::TRP1 | This study           |
| SSC2531       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 [YCplac117-pMET3-eCFP-TUB3::LEU2] [GFP::Rho1::URA3] | This study           |
| SSC2532       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 [YCplac117-pMET3-eCFP-TUB3::LEU2] [GFP::Rho1::URA3] | This study           |
| SSC2533       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 [YCplac117-pMET3-eCFP-TUB3::LEU2] [GFP::Rho1::URA3] rvs167-t::TRP1 | This study           |
| SSC2541       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 [YCplac117-pMET3-eCFP-TUB3::LEU2] [GFP::Rho1::URA3] | This study           |
| SSC2542       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 [YCplac117-pMET3-eCFP-TUB3::LEU2] [GFP::Rho1::URA3] rvs167-t::TRP1 | This study           |
| SSC2543       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 [YCplac117-pMET3-eCFP-TUB3::LEU2] [GFP::Rho1::URA3] | This study           |
amphiphysin function results in altered localization of the Rho1-GEF, Rom2 (Fig. 8). Potentially this would result in a failure to generate GTP bound Rho1 within the appropriate temporal-spatial window and a consequent failure to accumulate Rho1-GTP at the bud neck at the point of cell separation. The data presented in Fig. 9 demonstrate that this localization is completely abolished in the \textit{rvs167} null mutant. Both of these consequences, failure to properly localize Rom2 and Rho1, might stem from reduced levels of PIP2 at the bud neck that might be dependent upon Rvs167 plasma membrane binding analogous to one suggested role of BAR domain proteins in endocytic mechanisms [30]. Further support for this line of reasoning derives from recent evidence that has clearly demonstrated that budding yeast BAR domain proteins, including Rvs167, are able to organise stable, discrete PIP2 domains within the plasma membrane that allow for the assembly of plasma membrane associated protein complexes [64]. This interpretation does not exclude the possibility of direct interaction between the amphiphysin complex and either Rom2 or Rho1 although neither protein has previously been reported to interact with Rvs167 despite extensive interaction screens recorded in the \textit{Saccharomyces} Genome database. Importantly though, to the best of our knowledge, these data represent the first

### Table 1. Cont.

| Strain number | Genotype                                                                                                           | Source               |
|---------------|-------------------------------------------------------------------------------------------------------------------|----------------------|
| SSC1890       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 1 trp-1-1 can1-100 [YCplac33-pMET3-eCFP-TUB3::URA3] Cyk3-GFP::KanMX      | This study           |
| SSC2146       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 1 trp-1-1 can1-100 [YCplac33-pMET3-eCFP-TUB3::URA3] Cyk3-GFP::KanMX rvs167::TRP1 | This study           |
| SSC2868       | MATa ade2-1 his3-11,15 ura3-1 1 trp-1-1 can1-100 leu2-3,112;pmET3::Yiplocac128-pMET3-eCFP-TUB3::LEU2 HOF1-GFP::KanMX6 | This study           |
| SSC2870       | MATa ade2-1 his3-11,15 ura3-1 1 trp-1-1 can1-100 leu2-3,112;pmET3::Yiplocac128-pMET3-eCFP-TUB3::LEU2 HOF1-GFP::KanMX6 rvs167::TRP1 | This study           |
| SSC2487       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 1 trp-1-1 can1-100 Rom2-GFP::natMX6                                       | This study           |
| SSC2662       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 1 trp-1-1 can1-100 Rom2-GFP::natMX6 rvs167::TRP1                         | This study           |
| SSC2664       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 1 trp-1-1 can1-100 Rom2-GFP::natMX6 iqg1-1                               | This study           |
| SSC2484       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 1 trp-1-1 can1-100 Tus1-GFP::natMX6 iqg1-1                              | This study           |
| SSC2649       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 1 trp-1-1 can1-100 Tus1-GFP::natMX6 rvs167::TRP1                       | This study           |
| SSC2651       | MATa ade2-1 his3-11,15 leu2-3,112 ura3-1 1 trp-1-1 can1-100 Tus1-GFP::natMX6 iqg1-1                               | This study           |

### Table 2.

| Plasmid number | Plasmid and (construction)                     | Source               |
|----------------|-----------------------------------------------|----------------------|
| DNA425         | Yiploc128-pMET3::LEU2                         | Laboratory collection |
| DNA465         | pFA6a-13MYC::kanMX6                           | [57]                 |
| DNA614         | pFA6a-tdTomato::kanMX6                        | Iain Hagan           |
| DNA615         | pFA6a-tdTomato::phpMX6                        | Iain Hagan           |
| DNA637         | pFA6a-GFP(S65T)::natMX6                       | [59]                 |
| DNA669         | YCplac33-pMET3-eCFP-TUB3::URA3                | This study           |
| DNA679         | Yiploc128-pMET3-RVS167-13MYC::LEU2            | This study           |
| DNA689         | pFA6a-VC155::kanMX6                           | [60]                 |
| DNA690         | pFA6a-VN173::His2::MX6                        | [60]                 |
| DNA734         | Yiploc211-pGAL1-eCFP-TUB3::URA3               | This study           |
| DNA317         | pYEURA3::GAL1,10-MLC1::URA3                   | Laboratory collection |
| DNA713         | pYES2.D-GAL1-8SP1::URA3                       | This study           |
| DNA484         | Yiploc181-pMET3-8SP1::LEU2                    | This study           |
| DNA667         | Yiploc211-pMET3-eCFP-TUB3::URA3               | This study           |
| DNA619         | pFA6a-tdphMX6                                 | [59]                 |
| DNA463         | pFA6a-GFP(S65T)::kanMX6                       | [57]                 |
| DNA618         | pFA6a-natMX6                                  | [59]                 |
| DNA754         | GFP-RHO1::URA3                                | SP301 [29]           |
| DNA756         | GFP-RHO1[7::URA3]                             | SP307 [29]           |
| DNA666         | Yiploc128-pMET3-eCFP-TUB3::LEU2               | This study           |

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demonstration of an amphiphysin complex acting upstream in the activation of a Rho family GTPase.

In conclusion, the amphiphysin complex has two major functions during cytokinesis and cell separation in budding yeast as summarised in Fig. 9E, firstly, an auxiliary role in CAR assembly during early mitosis that requires the SH3 domain and is redundant with Hof1 [50]. Secondly, when CAR contraction is compromised, the amphiphysin polarity establishment function is required for the correct localization of at least three proteins, Rom2, Chs3 and GTP-bound Rh1 that function at the bud neck during cytokinesis and cell separation. The combination of failed CAR contraction and perturbation of polarity establishment leads to synthetic lethality. That Rvs167 is involved in both processes may serve to increase co-ordination between them, with subsequent gains in efficiency. The clear redundancy exhibited, both within and between CAR assembly and septation/cell separation, imply that CAR contraction itself might play a role in polarity establishment. Further, it is likely that this redundancy reflects evolution of robustness in the process of cytokinesis and cell separation.

Materials and Methods

Yeast strains, methods, and media

The yeast strains used in this study were isogenic with W303a and are listed in Table 1. Growth was in YEPD at the stated temperature [55]. Strains for fluorophore visualization were grown in complete or selective synthetic medium [55]. Solid media was made to the same composition, but with the addition of 2% agar. Salt sensitivity was tested by the addition of 6% sodium chloride. For positive selection of strains harbouring the drug resistance markers kanMX6, hphMX6 or natMX6 YEPD agar was supplemented with 220 μg/ml G418 disульфат (Melford), 300 μg/ml hygromycin B (InvivoGen), or 100 μg/ml nourseothricin (Werner Bioagents) respectively. Yeast transformations were carried out by a standard lithium acetate method [56]. GAL-CDC20, cdc20Δ cells were grown to exponential phase in SC Gal/Raf (as above, but substituting 2% galactose and 4% raffinose for glucose), before being transferred to SC containing 2% glucose to induce metaphase arrest. Release from the arrest was achieved by washing cells in 10 culture volumes of selective SC Gal/Raf and then resuspending cells in 1 volume of SC Gal/Raf. Induction of MET3 promoters was performed by growing cells in selective synthetic media lacking methionine. Repression of MET3 promoters was achieved by addition of sterile methionine to cultures (at a final amount of 50 mg/litre).

Plasmid construction

Standard DNA manipulations were employed for plasmid construction [56] with all DNA modifying enzymes used according to manufacturers specifications. The Escherichia coli strains, TOP10 (Invitrogen) or DH5α (Bioline) were used as a host for plasmid amplifications. Bacterial strains were grown in 2×TY media and frozen competent cells transformed according to manufacturers instructions. Plasmids used in this study are listed in Table 2.

Epitope tagging and strain construction

Epitope tagging was carried out by standard PCR amplification and subsequent homologous recombination into the yeast genome at the endogenous locus [57]. Further tagging and deletion of genes was similarly achieved using the plasmids pFA6a-GFP-kanMX6, pFA6a-HIS3-kanMX6, pFA6a-GFP-kanMX6, pFA6a-YFP-kanMX6, pFA6a-hphMX6, pFA6a-VC155-kanMX6 and pFA6a-VN173::HIS3MX6 as templates with appropriate primers [57,58,59,60]. Predicted sites of integration at the endogenous locus were confirmed by PCR testing of linkage of the drug resistant marker to flanking genomic DNA sequences. Yeast genomic DNA was isolated by a standard protocol [61]. All relevant DNA primer sequences are available on request.

In order to create the methionine regulated RVS167-13myc strains the RVS167 was first epitope tagged by standard methodologies. The RVS167-13MYC allele was then amplified by PCR and inserted into pCR-Blunt II TOPO (Invitrogen) according to manufacturer’s instructions. RVS167-13MYC was excised as a XhoI/BglII fragment and ligated into SacI/BamHI cut YIplac128-RVS167 13MYC (this work) to generate YIplac128-pMET3-RVS167-13MYC.

The plasmid was linearized by restriction digest with XcmI and integrated at the LEU2 locus to create the strain SSC1767. Subsequently the endogenous RVS167 was deleted by gene replacement with TRP1 to create SSC1754. An identical approach was used to create the integrated, methionine regulated RVS161-13myc allelic strains.

Preparation and staining of fixed cells for epifluorescence microscopy

Actin staining: 1×10⁶ formaldehyde fixed cells harvested by centrifugation at 4,000 rpm, resuspended in 25 μl of PBS solution containing 3 μm TRITC-conjugated phalloidin (Sigma) and incubated in the dark at room temperature for 90 minutes [62]. Nuclear staining: cells were harvested as above and resuspended in an appropriate volume of Vectashield with DAPI (Vector Laboratories). Cells were then either stored at 4°C or prepared immediately for microscopy.

Microscopy: image acquisition and analysis

Cells were prepared for live cell microscopy and images acquired and analysed as previously described [63].

Supporting Information

Figure S1 Rvs167-13Myc is functional. Indicated strains were grown to mid-log phase in media lacking methionine before plating onto indicated media and grown at temperature shown before imaging. A. Ectopic Rvs167-Myc expression rescues rv167Δ igg1-1 synthetic lethality (48 h growth). B. Ectopic Rvs167-Myc expression rescues salt sensitivity associated with rv167Δ (128 h growth). C. Rvs167-Myc expression rescues actin polarity defects associated with rv167Δ. Cells with ≥80% of actin patches localised in the bud or immediately adjacent at the bud neck were scored as polarised (between 94–145 cells scored at each time point). Shown are representative images from −MET (T = 0) of WT cells, with polarised actin patches, and rv167Δ cells with polarised (−MET) and depolarised (+MET) patches respectively. cV = ‘empty’ control vector. Scale bars = 2.5 μm. D. Ectopic Rvs161-Myc expression rescues rv161Δ igg1-1 synthetic lethality. E. Ectopic Rvs161-Myc expression only partially restores rv161Δ salt sensitivity after 128 h growth. (TIF)

Figure S2 Cyk3 and Hof1 behaviour is unaltered in the absence of amphiphysin function. A. CYK3-GFP localisation is unaltered in rv167Δ cells as shown by representative average intensity fluorescent images (scale bars = 2 μm). Maximum projected reconstructions of pixel data at the bud neck demonstrate Cyk3-GFP localises at ring and septal structures (scale bars for reconstructions = 1 μm). B. Quantification of Cyk3-GFP localization in large budded anaphase WT (n = 111), rv167Δ (n = 66), rv161Δ (n = 99), rv167Δ rv161Δ (n = 106) cells (left
Figure S3  

**Tus1-GFP dynamics are normal in iqg1-1 and rvs167Δ mutants.** WT (A), iqg1-1 (B) and rvs167Δ (C) cells expressing Tus1-GFP were imaged at 1 minute intervals (3 second exposures, 18 z-sections, 0.2 μm z-spacing) for 35 minutes. Maximum intensity projections of deconvolved z-stacks are shown for indicated time points. (TIF)

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**Author Contributions**

Conceived and designed the experiments: MJC CP. Performed the experiments: MJC. Analyzed the data: MJC CP. Contributed reagents/materials/analysis tools: MJC. Wrote the paper: MJC CP.

**Amphiphysin Function in Cytokinesis**

Graph and table RTE (n = 130), rvs167Δ (n = 173), rvs167Δ (n = 140), rvs167Δ rvs167Δ (n = 135) cells (right graph). (C) Hof1-GFP localisation is unaltered in rvs167Δ cells. Fluorescent panels represent average intensity projections (scale bars = 2.5 μm), except bud neck reconstructions (maximum projections, scale bars 1 μm). (C) DAPI staining of rvs167Δ cells in anaphase and telophase and to septa in late telophase. Cyk3-GFP (A) and Hof1-GFP (C) both form split bands either side of the division site in late telophase (arrowheads). (TIF)
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