The Transcriptomic Signature of RacA Activation and Inactivation Provides New Insights into the Morphogenetic Network of Aspergillus niger

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Abstract

RacA is the main Rho GTPase in Aspergillus niger regulating polarity maintenance via controlling actin dynamics. Both deletion and dominant activation of RacA (RacG18V) provoke an actin localization defect and thereby loss of polarized tip extension, resulting in frequent dichotomous branching in the ΔracA strain and an apolar growing phenotype for RacG18V. In the current study the transcriptomics and physiological consequences of these morphological changes were investigated and compared with the data of the morphogenetic network model for the dichotomous branching mutant ramosa-1. This integrated approach revealed that polar tip growth is most likely orchestrated by the concerted activities of phospholipid signaling, sphingolipid signaling, TORC2 signaling, calcium signaling and CWI signaling pathways. The transcriptomic signatures and the reconstructed network model for all three morphology mutants (ΔracA, RacG18V, ramosa-1) imply that these pathways become integrated to bring about different physiological adaptations including changes in sterol, zinc and amino acid metabolism and changes in ion transport and protein trafficking. Finally, the fate of exocytic (SncA) and endocytic (AbpA, SlbA) markers in the dichotomous branching mutant ΔracA was followed, demonstrating that hyperbranching does not per se result in increased protein secretion.

Introduction

Filamentous fungi such as Aspergillus niger are widely used in biotechnology for the production of various proteins, enzymes, food ingredients and pharmaceuticals [1–4]. During recent years, A. niger became an industrial model fungus, due to its well annotated genome sequence, sophisticated transcriptomics and proteomics technologies and newly established gene transfer systems allowing efficient and targeted genetic and metabolic engineering approaches [3,5–7].

The morphology of filamentous fungi strongly affects the productivity of industrial fermentations [8–10]. Basically, Aspergilli and all other filamentous fungi grow either as pellets or as freely dispersed mycelia during submerged growth. Both macro-morphologies depend among other things on hyphal branching frequencies – pellets are formed when hyphae branch with a high frequency, dispersed mycelia are a result of low branching frequencies. Whereas the formation of pellets is less desirable because of the high proportion of biomass in a pellet that does not contribute to product formation, long, unbranched hyphae are sensitive to shear forces in a bioreactor. Lysis of hyphae and the subsequent release of intracellular proteases have thus a negative effect on protein production. Hence, from an applied point of view, the preferred fungal macromorphology would consist of dispersed mycelia with short filaments derived from an optimum branching frequency. It is generally accepted that protein secretion occurs mainly at the hyphal apex [11–14]. Some studies suggested a positive correlation between the amount of hyphal branches and protein secretion yields [13,15–17], whereas other reports demonstrated no correlation [9,10]. Therefore, it is still a matter of debate whether a hyperbranching production strain would considerably improve protein secretion rates.

Different mutations can lead to a hyperbranching phenotype in filamentous fungi. For example, dichotomous branching (tip splitting) is a characteristic of the actin (act) and actinin mutants in Neurospora crassa and A. nidulans [19,20], a consequence of deleting the formin SepA in A. nidulans [21] or the polarisome component SpaA in A. nidulans and A. niger [22,23] and a consequence of inactivating the Rho GTPase RacA or the TORC2 complex component RmsA protein in A. niger [24,25]. Common to these different gene mutations is not only the phenotype they provoke but that they also disturb the dynamics of...
the actin cytoskeleton. Actin is crucial for polarized hyphal growth in filamentous fungi and controls many cellular processes, including intracellular movement of organelles, protein secretion, endocytosis and cytokinesis [26,27].

We have recently analyzed the function of all six Rho GTPase encoded in the genome of A. niger (RacA, CtaA, RhoA, RhoB, RhoC, RhoD) and uncovered that apical dominance in young germlings and mature hyphae of A. niger is predominantly controlled by RacA [24]. Both RacA and CtaA are not essential for A. niger (in contrast to RhoA) but share related functions which are executed in unicellular fungi only by Cdc42p [24]. The data showed that RacA localizes to the apex of actively growing filaments, where it is crucial for actin distribution. Both deletion and dominant activation of RacA (RacA<sup>G12V</sup> expressed under control of the maltose-inducible glucoamylase promoter gla<sub>D</sub>) provoke an actin localization defect and thereby loss of polarized tip extension. In the case of RacA inactivation, actin becomes hyperpolarized, leading to frequent dichotomous branching. Dominant activation of RacA, however, causes actin depolarization, leading to a swollen-tip phenotype and the formation of bulbous lateral branches. Interestingly, the dichotomous branching phenotype suggested that loss of apical dominance in Δrac<sub>D</sub> can frequently be overcome by the establishment of two new sites of polarized growth. This phenotype resembles the phenotype of the ramosa-1 mutant of A. niger, which harbors an temperature-sensitive mutation in the TORC2 component RmsA causing a transient contraction of the actin cytoskeleton [25,28,29].

Altogether, the data supported the model that RacA is important to stabilize polarity axes of A. niger hyphae via controlling actin (de)polymerization at the hyphal apex. The aim of the present study was to unravel the genetic network into which RacA is embedded and which, when disturbed due to deletion or dominant activation of RacA, leads to loss of polarity maintenance and in the case of Δrac<sub>D</sub>, to reestablishment of two new polarity axes. To determine whether the hyperbranching phenotype of Δrac<sub>D</sub> leads to an increase in the amount of secreted proteins, the transcriptomes of our previously established RacA mutant strains (Δrac<sub>D</sub>, P<sub>glam</sub>Δ::racD<sup>G12V</sup>) were compared with the transcriptomes of the respective reference strains (wt, P<sub>gmaltose</sub>::<sup>D</sup>racA). By applying defined culture conditions in bioreactor cultivations, branching morphologies as well as physiological parameters including specific growth rate and protein production rate were characterized. Finally, the implication of Δrac<sub>D</sub> on endocytosis and exocytosis in A. niger was examined by analyzing reporter strains harboring fluorescently tagged SlaB and Abp<sub>A</sub> (markers for endocytotic actin) and SncA (marker for secretory vesicles). The data obtained were compared with transcriptomic and physiological data of the dichotomous branching mutant ramosa-1 [25], thereby providing new insights into the morphogenetic network of A. niger.

Results

Physiological consequences of RacA inactivation

As previously reported, deletion of rac<sub>D</sub> in A. niger provokes hyperbranching germ tubes and hyphae, which are shorter in length but wider in hyphal diameter. This frequent branching results on solid medium in a more compact colony with a reduced diameter due to slower tip extension rates [24]. In order to further characterize the implications of loss of RacA function, the reference strain (wild-type N402) and the Δrac<sub>D</sub> strain were cultivated in triplicate batch cultures using maltose as growth-limiting carbon source. Propagation of both strains gave rise to homogeneous cultures of dispersed mycelia, whereby loss of RacA resulted in an about 30% higher branching frequency (Fig. 1 and Table 1). Physiological profiles including growth curves, maximum specific growth rates and specific protein secretion rates were obtained with high reproducibility and were nearly identical for both strains despite the significant difference in their morphology (Fig. 2 and Table 2). This result might come with surprise because of the negative effect of the rac<sub>D</sub> deletion on radial colony growth on solid medium [24]. However, growth on solid media can only be assessed based on colony diameter (reflecting tip extension) and not on biomass accumulation (i.e. increase in cell volume per time). During exponential growth, growth yield on substrate (N<sub>x</sub>Y<sub>x</sub>) was comparable in both strains; 0.63±0.03 and 0.60±0.02 gm<sub>biomass</sub> g<sub>substrate</sub><sup>-1</sup> for Δrac<sub>D</sub> and N402, respectively. Notably, the amount of extracellular protein was not altered in Δrac<sub>D</sub> strain compared to N402 (Table 2). Hence, an increased branching frequency is the only highly significant consequence of rac<sub>D</sub> disruption, which, however, does not per se result in higher protein secretion rates.

Consequences of RacA inactivation on exo- and endocytosis

We previously showed that RacA is important for actin localization at the hyphal tip [24]. As actin is important for both exo- and endocytosis, the consequences of rac<sub>D</sub> deletion on both was assessed in A. niger by following the localization of fluorescently-labelled reporter proteins SncA, Abp<sub>A</sub> and SlaB, respectively. SncA is the vesicular-SNARE that is specific for the fusion of Golgi derived secretory vesicles with the plasma membrane [30] and used as marker for exocytosis in A. nidulans [31,32], Abp<sub>A</sub>/Abp<sub>A</sub> and Sla2/SlaB are actin binding proteins and well characterized endocytotic markers in yeast and filamentous fungi [31,33,34]. Screening of the genome sequence of A. niger [5] predicted for each of the established marker proteins a single orthologue for A. niger: An12g07570 for SncA, An03g06960 for Abp<sub>A</sub>/Abp<sub>A</sub> and An11g10320 for Sla2/SlaB.

We constructed a reporter strain expressing a fusion of GFP with the v-SNARE SncA as described elsewhere (Kwon et al., manuscript submitted). In brief, physiological expression levels of GFP-SncA was ensured by fusing GFP between the N-terminus and the promoter of snc<sub>D</sub> and used this cassette to replace it with the endogenous snc<sub>D</sub> gene (giving strain FG7). As depicted in Figure 3A, GFP-SncA is visible as punctuate intracellular structures representing secretory vesicles. These vesicles accumulate towards the hyphal tip, overlap with the Spitzenkorper and are highest at the extreme apex, which is proposed to be the site of exocytosis in filamentous fungi [31]. This localization of GFP-SncA in A. niger was very similar to the localization previously reported for other filamentous fungi [14,31,35–37]. Importantly,
the amount of secretory vesicles per individual hyphal tip was affected in the ΔracA strain. Although the localization of GFP-SncA was similar to the wild-type strain, the intensity of the signal was considerably lower. Quantification of the GFP signal intensities in both strains revealed that the tips of wild-type hyphae display a GFP-SncA gradient of ~20–25 μm but only about ~10 μm in the racA deletion strain (Fig. 3B). Both strains, however, do not differ in their specific protein secretion rates (Table 2), implying that the total amount of secretory vesicles is the same in both strains. This discrepancy can most easily be explained by the assumption that secretory vesicles in the hyperbranching ΔracA strain are merely distributed to more hyphal tips, which in consequence lowers the amount of vesicles per individual hyphal tip.

To follow the effect of racA deletion on endocytosis, AbpA and SlaB were labeled using a C-terminal labeling strategy as previously reported for A. nidulans [31]. Importantly, both AbpA-CFP and SlaB-YFP were expressed at physiological levels by using the respective endogenous promoter and by replacing the constructs with the endogenous abpA and slaB gene, respectively (Fig. 4B). AbpA-CFP (strain MK6.1) and SlaB-YFP (strain MK5.1) transformants were phenotypically indistinguishable from the recipient strain, indicating that both AbpA-CFP and SlaB-YFP are functionally expressed (Fig. 4 and data not shown). Although AbpA-CFP and SlaB-YFP fluorescence signals were only weakly detectable (which is a direct consequence of their low endogenous expression level), both proteins were visible in the wild-type background as peripheral punctate structures and formed a subapical ring likely reflecting the endocytic machinery (Fig. 4A–C). Fluorescent signals were excluded from the hyphal apex which is in agreement with previous reports for A. nidulans showing that endocytosis occurs behind the tip [31,34]. The signal of SlaB-YFP but not AbpA-CFP seemed to be intimately associated with the plasma membrane (data not shown), which would be in agreement with the function of both proteins - Sla2/SlaB is involved in early endocytic site initiation while Abp1/AbpA is important for invagination, scission and release of endocytotic vesicles [38]. SlaB-YFP and AbpA-CFP fluorescence was also occasionally observed at septa or sites destined for septum formation (Fig. 4D), probably suggesting an involvement of endocytotic events at septa as recently reported for A. oryzae [14]. Importantly, the intensity

| Table 2. Physiological characterization of N402 and ΔracA strains. |
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| **N402** | **ΔracA** |
| Maximum specific growth rate (h⁻¹) | 0.22±0.01 | 0.24±0.01 |
| Yield (g dry. g maltose⁻¹) | 0.60±0.02 | 0.63±0.03 |
| Respiratory quotient (RQ) | 0.97±0.05 | 1.03±0.03 |
| Acidification (mmol base g dry.⁻¹ h⁻¹) | 1.19±0.06 | 1.17±0.02 |
| Specific protein secretion rate (mg protein g dry.⁻¹ h⁻¹) | 0.49±0.07 | 0.53±0.02 |

Biomass samples were taken from triplicate independent batch cultivations using maltose as carbon source (Fig. 2). Mean values ± standard deviations are given. No significant difference was observed with any of the variables (two tailed t-test, p<0.01). RQ, respiratory quotient calculated as the ratio of CO₂ production and O₂ consumption rates.

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and distribution of Slab-YFP and AbpA-CFP differed slightly in the wild-type and the \( \Delta \text{racA} \) strain (Fig. 4E–G). The endocytotic actin ring as visualized by AbpA-CFP was sharper formed in the wild-type background but more diffuse in the \( \Delta \text{racA} \) strain, an observation which, however, was not so evident for Slab-YFP (Fig. 4F–G). Still, the endocytotic ring formed by both Slab-YFP and AbpA-CFP seemed to be positioned closer to the hyphal apex in the \( \Delta \text{racA} \) strain (Fig. 4G), suggesting that deletion of RacA affected endocytotic processes and provoked a slight mislocalization of the endocytotic ring.

The transcriptomic fingerprint of hyperbranching in \( \Delta \text{racA} \)

To study the transcriptomic consequences due to deletion of racA, RNA samples from triplicate bioreactor cultivations were taken after both wt and \( \Delta \text{racA} \) cultures reached the mid-exponential growth phase (biomass concentration 3.7 gr kg\(^{-1}\)). A total of 139 genes out of 14,165 \( \textit{A. niger} \) genes were identified as differentially expressed, 44 of which displayed increased and 95 genes decreased expression levels in \( \Delta \text{racA} \) (FDR, 0.05). The complete list of differentially expressed genes, including fold change and statistical significance is given in Tables S1 and S2.

Interestingly, the modulated gene set in \( \Delta \text{racA} \) is very small (139 genes, i.e. 1% of all \( \textit{A. niger} \) genes) but in the same range as the differentially expressed gene set in the dichotomous branching mutant \( \textit{ramosa-1} \) of \( \textit{A. niger} \) (136 genes; [25]). Although the affected gene sets had opposite signs (44 up/95 down in \( \Delta \text{racA} \), 109 up/27 down in \( \textit{ramosa-1} \)), similar processes were affected in both hyperbranching strains (Table 3): (i) (phospho)lipid signaling, (ii) calcium signaling, (iii) cell wall integrity (CWI) signaling and cell wall remodeling, (iv) \( \gamma \)-aminobutyric acid (GABA) metabolism and (v) transport phenomena. Specific responses for \( \Delta \text{racA} \) but not \( \textit{ramosa-1} \) included genes related to actin localization and protein trafficking.

In the case of (phospho)lipid signaling, genes encoding enzymes for the synthesis of the key regulatory lipid molecules diacylglycerol (DAG) and inositolpyrophosphates (IP6 and IP7) were differentially expressed in both \( \Delta \text{racA} \) and \( \textit{ramosa-1} \). These molecules play important roles in the regulation of actin polarisation, CWI and calcium signaling in lower and higher eukaryotes (see discussion). Notably, expression of \( \textit{An}04g03870 \) predicted as ortholog of the \( \textit{S. cerevisiae} \) Dpp1p (DAG pyrophosphate phosphatase) is affected in both strains; however, down-regulated in \( \Delta \text{racA} \) but up-regulated in \( \textit{ramosa-1} \). The same opposite response was observed for two \( \textit{A. niger} \) ORFs predicted to encode inositol hexaki-/heptaki-phosphate kinases synthesizing the signaling molecules inositol polyphosphates IP6 or IP7: \( \textit{An}14g04590 \) (Ksc1p ortholog) is down-regulated in \( \Delta \text{racA} \), whereas \( \textit{An}16g05020 \) (Vip1p ortholog) is up-regulated in \( \textit{ramosa-1} \). Inositol polyphosphates IP4-IP6 are known to bind to the C2B domain of the calcium sensor protein synaptotagmin [39]. This binding inhibits exocytosis of secretory vesicles, whereas binding of calcium to the C2A domain of synaptotagmin activates exocytosis [40]. In the \( \Delta \text{racA} \) strain, four calcium transporters are down-regulated compared to the wild-type situation, two of which (\( \textit{An}01g03100 \), \( \textit{An}05g00170 \) code for the ortholog of the \( \textit{S. cerevisiae} \) Vcx1p protein, which is also differentially expressed under hyperbranching conditions in \( \textit{ramosa-1} \) (Table 3). This observation hints at the possibility that reduced GFP-SncA fluorescence at \( \Delta \text{racA} \) hyphal tips is somehow linked with changes in IP6/IP7 and calcium levels in \( \Delta \text{racA} \), which would be in agreement with a recent report which demonstrated that calcium spikes accompany hyphal branching in \( \textit{Fusarium} \) and \( \textit{Magnaporthe} \) hyphae [41]. Changes in the intracellular calcium distribution also affect the homeostasis of other ions. Congruently, 12 genes putatively
encoding transport proteins for ions (Na⁺, K⁺, Fe²⁺) and small molecules (phospholipids, amino acids, peptides, hexoses) displayed differential transcription in ΔracA as also observed for ramosA-I (Table 3), suggesting that ion homeostasis and/or metabolic control systems are also important to maintain polar growth. Reduced exocytotic GFP-SncA signals at hyphal tips of the ΔracA strain would imply that less cell wall biosynthetic/remodeling enzymes are transported to the tip. Indeed, expression of ten ORFs encoding cell membrane and cell wall genes and three ORFs involved in protein trafficking were down-regulated in ΔracA (Table 3). Whatever the consequences of reduced expression of cell wall or ion homeostasis genes are, none of these changes led to increased sensitivity of the ΔracA strain towards cell wall stress agents (calciofluor white), different salts (MgCl₂, KCl, NaCl) or oxidative conditions (H₂O₂, menadion; data not shown), suggesting that the integrity of the cell wall or cell membrane is not disturbed in the ΔracA strain.

Inactivation of RacA, however, has considerable consequences on actin localization as previously reported [23]. Congruently, five ORFs involved in actin polarization were down-regulated in ΔracA strain (Table 3). Of special importance is the polarisomal component SpaA, whose deletion has been shown to cause a hyperbranching phenotype and reduced growth speed in A. niger [23] and two ORFs which are homologous to the S. cerevisiae amphyphysin-like proteins Rvs161p and Rvs167p. The latter function as heterodimer in S. cerevisiae, bind to phospholipid membranes and have established roles in organization of the actin cytoskeleton, endocytosis and cell wall integrity [42].

The transcriptomic fingerprint of apolar growth in RacAG18V

Next, we wished to dissect the transcriptomic adaptation of A. niger to dominant activation of RacA. Batch cultures of our previously established RacA mutant strains PgalA:racAG18V and PgalA:racA (reference strain) were started with xylose (0.75%) as repressing carbon source. After the cultures reached the exponential phase and xylose was consumed, maltose (0.75%) was added to induce expression of genes under control of PgalA. Hypothetically, a so far unknown RacA-dependent GAP ensures that the activity of RacA is spatially restricted to the hyphal apex in the PgalA-racA strain thereby maintaining a stable polarity axes even under inducing conditions (Fig. 5). However, this control mechanism is leveraged off in the PgalA-racAG18V mutant strain, as the GTPase-negative G18V mutation traps RacA in its active, GTP-bound form [24]. Hence, the switch from xylose to maltose leads to a loss of polarity maintenance in the PgalA-racAG18V strain and the formation of clavate-shaped hyphal tips and bulbous lateral branches (Fig. 5). RNA samples were extracted from duplicate cultures 2 and 4 h after the maltose shift and used for transcriptomic comparison. Expression of 3,757 (506) genes was modulated after 2 h (4 h) of induction, 1,906 (282) of which showed increased and 1,851 (224) decreased expression levels in the PgalA-racAG18V strain when compared to the PgalA-racA reference strain (FDR <0.05). The complete list of differentially expressed genes, including fold change and statistical significance is given in Tables S1 and S2. GO enrichment analysis using the FETGOat tool [43] discovered that most of these genes belong to primary metabolism, suggesting that both strains differed in their ability to quickly adapt to the new carbon source (Table S3).

To identify those genes which only relate to the difference between polar to apolar morphology in PgalA-racAG18V but are independent from time and carbon source, a Venn diagram was constructed (Fig. 6) and the intersection determined representing genes which are differently expressed after both 2 and 4 h in PgalA-racAG18V when compared to the 2 h and 4 h data sets of the PgalA-racA reference strain. Overall, 148 genes showed different expression, 106 of which were up-regulated and 42 of which were down-regulated in PgalA-racAG18V (Table S4). Again, only a small set of genes (about 1% of the A. niger genome) show different expression levels during polar and apolar growth. Table 4 highlights the most interesting genes of this compilation, which could be grouped into several regulatory processes including (i) (phospho)lipid signaling, (ii) calcium signaling, (iii) CWI signaling and (iv) nitrogen signaling. In addition, metabolic processes including primary metabolism (amino acid biosynthesis) and secondary metabolism (polyketide synthesis, non-ribosomal peptide synthesis) were affected as well.

The transcriptomic fingerprint indicated that the turgor pressure is increased during apolar growth and thereby an osmotic and cell wall stress is sensed in the PgalA-racAG18V strain. An14g02970, an ORF with strong similarity to the Snl1p histidine kinase osmosensor of S. cerevisiae which forms a phosphorelay system to activate the Hog1 MAP kinase cascade [44], showed increased expression. In agreement, some cell wall genes which have been shown to respond to caspofungin-induced cell wall stress in A. niger [45] were up-regulated as well: agtA, a GPI-anchored alpha-glucanosyltransferase and two putative cell wall protein encoding genes phil4 and An12g10200. In addition, other proteins, known to be up-regulated under cell wall and osmotic stress in S. cerevisiae showed enhanced expression in PgalA-racAG18V: An16g02850 (ortholog of the citrin transglycosylase Crl1p), [46], An02g05490 (Ca²⁺/calmodulin dependent protein kinase Cmk2p, [47] and An07g01250 (ortholog of the multidrug transporter Pdr5p, [48]. Furthermore, increased expression was also observed for An03g06500 encoding an ortholog of the plant zeaxanthin epoxidase, which catalyses one step in the biosynthetic pathway of the plant hormone abscisic acid, known to protect plant cells against dehydration under high-salinity stress [49].

The expression of many transporters and permeases (iron, hexosamines, amino acids and peptides) was also modulated in PgalA-racAG18V as well as the expression of several amino acid biosynthetic genes, most of which were down-regulated (lysine, arginine, threonine, methionine; Table 4). As also some ORFs predicted to function as important activators in replication (An03g06930, An08g07090) and translation (An18g04650) displayed decreased expression suggests that reduced tip extension during apolar growth slows down basic cellular processes. In this context, it is interesting to note that An01g09260 predicted to break down sphingosin 1-phosphate (S1P) showed increased...
Table 3. Selected genes whose expression profile respond to hyperbranching in ΔracA (this work) and ramosa-1 [25]. Genes are ordered into different processes and functions.

| Predicted protein function* | ΔracA versus wt |  | ramosa-1 versus wt |  |
|----------------------------|----------------|-----------------------------|-----------------------------|
|                            | Open reading frame code | Up/Down | Closest S. cerevisiae ortholog | Open reading frame code | Up/Down | Closest S. cerevisiae ortholog |
| (Phospholipid metabolism and signaling) |                       |          |                          |                          |
| phosphatidyl synthase, synthesis of phosphatidyl alcohols | An04g04210 | ↑ | Erg6 | An02g08050 | ↑ |
| sterol 24-C-methyltransferase, ergosterol synthesis | An01g07000 | ↓ | Kcs1 | An16g05020 | ↑ |
| C-14 sterol reductase, ergosterol synthesis | An14g04590 | ↓ | Vip1 | An18g06410 | ↑ |
| inositol hexaki-/heptaki-phosphate kinase, synthesis of IP6, IP7 | An04g04210 | ↓ | Erg6 | An02g08050 | ↑ |
| plasma membrane protein promoting PI4P synthesis | An18g01090 | ↓ | Pib3 | An02g13220 | ↓ |
| phospholipase B, synthesis of glycerophosphocholine | An18g01090 | ↓ | Pib3 | An02g13220 | ↓ |
| phospholipase D, synthesis of phosphatidic acid | An15g07040 | ↑ | Spo14 | An02g01180 | ↓ |
| diacylglycerol pyrophosphate phosphatase, synthesis of DAG | An04g03870 | ↓ | Dpp1 | An04g03870 | ↑ |
| diacylglycerol pyrophosphate phosphatase, synthesis of DAG | An02g01180 | ↓ | Dpp1 | An11g05330 | ↑ |
| choline/ethanolamine permease | An16g01200 | ↑ | Hnm1 | An01g13290 | ↓ |
| choline/ethanolamine permease | An16g03050 | ↑ | Vcx1 | An01g03100 | ↓ |
| transcription factor important for sterol uptake | An12g00680 | ↓ | Upc2 | An01g03100 | ↓ |
| transcription factor important for sterol uptake | An07g05570 | ↑ | Kre2 | An04g0380 | ↑ |
| mannosyl-inositol phosphorylceramide (MIPC) synthase | An05g02310 | ↓ | Sur1 | An07g05570 | ↑ |
| Calcium homeostasis and signaling |                       |          |                          |                          |
| Ca²⁺/calmodulin dependent protein kinase | An02g05490 | ↑ | Cmk2 | An16g03050 | ↑ |
| Ca²⁺/calmodulin dependent protein kinase | An01g03100 | ↓ | Vcx1 | An05g00170 | ↑ |
| vacular Ca²⁺/H⁺ exchanger | An05g00170 | ↑ | Vcx1 | An01g03100 | ↓ |
| vacular Ca²⁺/H⁺ exchanger | An01g03100 | ↓ | Vcx1 | An05g00170 | ↑ |
| Ca²⁺/H⁺ exchanger | An01g03100 | ↓ | Vcx1 | An05g00170 | ↑ |
| Ca²⁺/ATPase | An19g00350 | ↓ | Pmc1 | An02g06350 | ↑ |
| Ca²⁺/phospholipid-transporting ATPase | An04g06840 | ↑ | Drs2 | An04g06840 | ↑ |
| Cell wall remodeling and integrity |                       |          |                          |                          |
| membrane receptor, CWI signaling | An01g14820 | ↑ | Wsc2 | An18g03740 | ↑ |
| MAP kinase kinase, CWI signaling, MkkA | An01g14820 | ↑ | Wsc2 | An18g03740 | ↑ |
| plasma protein responding to CWI signaling | An07g08960 | ↓ | Pun1 | An07g08960 | ↓ |
| plasma protein responding to CWI signaling | An08g01170 | ↓ | Pun1 | An08g01170 | ↓ |
| α-1,3-glucanase | An04g03680 | ↑ | An08g09610 | ↑ |
| β-1,3-glucanosyltransferase (GPI-anchored) | An16g06120 | ↑ | Gas1 | An16g06120 | ↑ |
| β-1,4-glucanase | An03g05530 | ↑ | An03g00530 | ↑ |
| chitin synthase class II, similar to ChsA of A. nidulans | An07g05570 | ↑ | Chh1 | An07g05570 | ↑ |
| chitin transglycosidase (GPI-anchored) | An07g01160 | ↓ | Crh2 | An13g02510 | ↑ |
| chitinase (GPI-anchored), similar to ChiA of A. nidulans | An09g06400 | ↓ | Cts1 | An09g06400 | ↓ |
| β-mannosidase | An11g06540 | ↑ | Dfg1 | An16g08090 | ↓ |
| endo-mannanase (GPI-anchored), DfgE | An16g08090 | ↓ | Dfg1 | An16g08090 | ↓ |
| α-1,2-mannosyltransferase | An14g03910 | ↑ | Kre2 | An14g03910 | ↑ |
| α-1,3-mannosyltransferase | An15g04810 | ↓ | Mnt2 | An15g04810 | ↓ |
| α-1,4-mannosyltransferase | An05g02320 | ↓ | Mnt2 | An05g02320 | ↓ |
| cell wall protein | An14g01840 | ↓ | Tir3 | An04g05550 | ↑ |
| cell wall protein | An11g01190 | ↓ | Sps22 | An11g01190 | ↑ |
| cell wall protein | An03g05560 | ↑ | Sps22 | An03g05560 | ↑ |
| cell wall protein | An04g03830 | ↑ | Sps22 | An04g03830 | ↑ |
| cell wall protein | An02g11620 | ↓ | Sps22 | An02g11620 | ↓ |
| Predicted protein function* | ΔracA versus wt | ramosa-1 versus wt |
|----------------------------|-----------------|-------------------|
|                            | Open reading frame code | Up/Down | Closest S. cerevisiae ortholog | Open reading frame code | Up/Down | Closest S. cerevisiae ortholog |
| plasma membrane protein    | An02g08030 | ▼ | Pmp3 |
| **GABA metabolism**         |                 |      |                |                 |      |                |
| glutaminase                 | An11g07960 | ▼ |                |                |      |                |
| γ-aminobutyrate transaminase| An17g00910 | ▼ | Uga1 |
| NAD(+) dependent glutamate dehydrogenase | An02g14590 | ▼ | Gdh2 |
| GABA permease              | An16g01920 | ▼ |                |                |      |                |
| transcription factor for GABA genes | An02g07950 | ▼ |                |                |      |                |
| **Transporter**            |                 |      |                |                 |      |                |
| mitochondrial phosphate translocator | An02g04160 | ▼ | Mir1 |
| mitochondrial ABC transporter during oxidative stress | An12g03150 | ▼ | Mdl1 |
| vacuolar glutathione S-conjugate ABC-transporter | An13g03230 | ▼ | Ycf1 |
| plasma membrane Na+/K⁺-exchanging ATPase alpha-1 chain | An09g00930 | ▼ | Opt1 |
| plasma membrane K⁺ transporter | An03g02700 | ▼ | Trk1 |
| multidrug transporter      | An01g05830 | ▼ | Qdr1 |
| low-affinity Fe(II) transporter of the plasma membrane | An16g06300 | ▼ | Fet4 |
| vacuolar H⁺-ATPase subunit, required for copper and iron homeostasis | An10g00680 | ▼ | Vma3 |
| siderophore-iron transporter | An12g05510 | ▼ | Taf1 |
| mitochondrial carrier protein | An14g01860 | ▼ | Rim2 |
| vacuolar zinc transporter  | An15g03900 | ▼ | Zrc1 |
| allantoin permease         | An18g01220 | ▼ | Dal5 |
| oligopeptide transporter   | An13g01760 | ▼ | Opt1 |
| oligopeptide transporter   | An11g01400 | ▼ | Opt1 |
| hexose/glucose transporter | An06g00560 | ▼ | Hxt13 |
| amino acid transporter     | An03g00430 | ▼ |                |
| galactose transporter      | An01g09790 | ▼ | Gal2 |
| FAD transporter into endoplasmatic reticulum, CWI-related | An01g09050 | ▼ | Fkc2 |
| **Protein trafficking**    |                 |      |                |                 |      |                |
| GTPase activating protein involved in protein trafficking | An01g02860 | ▼ | Gyp8 |
| t-SNARE protein important for fusion of secretory vesicles with the plasma membrane | An02g05390 | ▼ | Sec9 |
| vacuolar protein important for endosomal-vacuolar trafficking pathway | An11g01810 | ▼ | Rcr2 |
| **Actin localisation**     |                 |      |                |                 |      |                |
| polarsome component SpaA  | An07g08290 | ▼ | Spa2 |
| actin-binding protein involved in endocytosis | An03g01160 | ▼ | Lsb4 |
| protein required for normal localization of actin patches | An16g02680 | ▼ | Apd1 |
| Amphysin-like protein required for actin polarization | An17g01945 | ▼ | Rvs161 |
| Amphysin-like protein required for actin polarization | An09g04300 | ▼ | Rvs167 |
| **Other signaling processes** |                 |      |                |                 |      |                |
| Ser/Thr protein kinase important for K⁺ uptake | An17g01925 | ▼ | Sat4 |
| transcription factor for RNA polymerase II | An16g07220 | ▼ | Tfg2 |
| negative regulator of Cdc42 | An12g04710 | ▼ | Vtc1 |
| putative C2H2 zinc-finger transcription factor | An04g01500 | ▼ |      |
| SUN family protein involved in replication | An08g07090 | ▼ | Sim1 |
| similar to A. nidulans transcription factor RosA | An16g07890 | ▼ | Ume6 |
expression during apolar growth. S1P is a sphingolipid acting as second messenger in lower and higher eukaryotes regulating respiration, cell cycle and translation [50] and is also important for the sole sphingolipid-to-glycerolipid metabolic pathway [51]. As also expression of An02g06440, a predicted ortholog of the S. cerevisiae sphingosin flippase Rsb1p which extrudes sphingosin into the extracytoplasmic side of the plasma membrane, was increased during apolar growth might suggest that reduced levels of S1P are present in apolar growing hyphal tips (see discussion).

Interestingly, none of the extracted 148 genes (Table S4) showed any link to actin filament organization, although actin polarization is lost in the P glaA racAG18V strain and actin patches are randomly scattered intracellularly or at the cell periphery [24]. One explanation might be that transcription of actin-related genes was immediately altered after maltose addition which induced the switch from polar to apolar growth in P glaA racAG18V. To still get a glimpse on genes involved in actin patch formation, the 2 h dataset of P glaA racAG18V versus P glaA racA was screened for the presence of enriched GO terms related to actin (Table S3). Using this approach, 10 actin-related genes were extracted which are summarized in Table 5. Most interestingly, An18g04590, an ortholog of the S. cerevisiae Rho GDP dissociation inhibitor Rdi1p displayed increased expression. Rdi1p regulates the Rho GTPases Cdc42p, Rho1p and Rho4p, localizes to polarized growth sites at specific times of the cell cycle and extracts all three proteins from the plasma membrane to keep them in an inactive cytosolic state [52–54]. Overexpression of Rdi1p causes slightly rounder cell morphology in S. cerevisiae [55]. Another interesting actin-related gene showing increased expression in strain P glaA racAG18V is An11g02840, the predicted homolog of the S. cerevisiae Slm2 protein. Slm2p binds to the second messenger phosphatidylinositol-4,5-bisphosphate (PIP2) and to the TORC2 signaling complex and integrates inputs from both signaling pathways to control polarized actin assembly and cell growth [56]. In addition, it is also

### Table 3. Cont.

| Predicted protein function* | ΔracA versus wt | ramosa-1 versus wt |
|----------------------------|-----------------|--------------------|
|                            | Open reading    | Closest S.         |
|                            | frame code      | cerevisiae ortholog|
|                            | Up/Down         |                    |
| Others                     |                 |                    |
| hypothetical aspergillosis | An03g00770      | An03g00770         |
| allergen rAsp              | ↓                | ↑                  |

Genes up-regulated are indicated with ↑, genes down-regulated with ↓. Differential gene expression was evaluated by moderated t-statistics using the Limma package [82] with a FDR threshold at 0.05 [83]. Identical ORFs which are differentially expressed in both ΔracA and ramosa-1 are indicated in bold. Fold changes and statistical significance is given in Additional file 1 and 2.

*Protein functions were predicted based on information inferred from the Saccharomyces genome data base SGD (http://www.yeastgenome.org/) and the Aspergillus genome database AspGD (http://www.aspergillusgenome.org/).

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**Figure 5. Biomass accumulation and morphology during submerged cultivation of P glaA-racAG18V and P glaA-racA mutant strains.**

(A) Growth curve for both mutant strains. The dashed line indicates the time point when the inducing carbon source maltose was added. The two arrows indicate the time points at which samples for transcriptome analysis were taken. (B) Dispersed hyphal morphology at the time point of maltose addition as well as 2 and 4 h after maltose addition. Bar, 20 μm.

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messenger diacylglycerol (DAG), the activator of mammalian and fungal protein kinase C, which in fungi is a component of the CWI pathway localized upstream of the MAP kinase kinase Mkk1/2 (MkkA in A. niger; for review see [59]). Targets of the CWI signaling pathway are cell wall remodeling genes, five of which were differentially expressed in RacA hyper- or inactivation strains (Table 6). From these five genes, three are of special importance as they were also effector genes in the hyperbranching mutant ramosa-1 [25]. Although calcium signaling genes seemed not be among the extracted 38 genes, its indirect involvement might be conceivable. For example, An18g01090 encoding the predicted ortholog of the S. cerevisiae phospholipase B (Phb3p) is among this gene set. Phb3p is activated at high concentrations of Ca2+ and specifically accepts phosphatidylinositol as a substrate to keep its concentration on the outer membrane leaflet low [60]. Finally, 17 RacA effector genes encode proteins of unknown function, most of which have no predicted orthologs in S. cerevisiae. As their function, however, seems to be important for morphological changes in A. niger, they are highly interesting candidate genes for future analyses.

Discussion

The fungal actin cytoskeleton is highly dynamic and fulfills multiple functions important for cell polarity regulation, endocytosis, exocytosis and septation. Central regulators of actin polymerization and depolymerization are Rho GTPases whose activity is regulated by their membrane-cytosplastic shuttling which itself is modulated by external or internal morphogenetic signals. Actin dynamics is thus controlled by a network of signaling pathways that sense and integrate different stimuli [61]. We have recently proposed that the A. niger GTTPases RacA and CiaA (Cdc42p) can substitute each other with respect to actin assembly but that actin disassembly is mainly under control of RacA. A racA deletion mutant is thus not affected in actin polymerization (because is secured by CiaA) but impaired in actin disassembly. In consequence, maintenance of apical dominance can become frequently lost in the racA deletion strain resulting in a hyperbranching phenotype. In contrast, RacA trapped in its active, GTP-bound form (RacA GTP) provokes the formation of higher-order actin structures, i.e. actin patches, which cause loss of polarity maintenance and the formation of round, apolar growing cells [24]. The purpose of the current study was to identify the transcriptional signature associated with morphological changes in hyphal tip growth of A. niger. The transcriptional response of A. niger provoked by inactivation and hyperactivation of RacA, respectively, was determined and compared with the transcriptomic fingerprint of the apical branching transcriptome of the ramosa-1 mutant [25]. The data obtained allowed us to reconstruct the transcriptomic network that helps A. niger to adapt to abnormal morphologies and to secure the integrity of its cell wall.

A transcriptomic perspective on the morphogenetic network of A. niger

A central result of our comparative transcriptomics approach is the finding that several lipid molecules are likely involved in the maintenance of polar growth in A. niger (Fig. 7). The synthesis of important phospho- and sphingolipid molecules (phosphatidic acid, DAG, PIP2, inositol phosphates [IP], glycerophosphocholine, mannose-inositol-phosphoceramide (MIPC) and S1P seem to be modulated during apical branching (ΔracA, ramosa-1) and apolar growth (PglA-RacA ΔGTP), as genes encoding respective synthetic or degrading enzymes showed differential expression in comparison to the wild-type (Tables 3, 4 and 6). Many of these molecules
### Table 4. Selected genes whose expression profiles differ between polar and apolar growth in *PglA-racA<sup>GR8V</sup>* versus *PglA-racA* in a time- and carbon source-independent manner. Genes are ordered into different processes and functions.

| Predicted protein function* | Open reading frame code | Up/Down | Closest *S. cerevisiae* ortholog |
|----------------------------|-------------------------|---------|----------------------------------|
| **(Phospho)lipid metabolism and signaling** |                         |         |                                  |
| dehydrogenase involved in sphingosin 1-phosphate breakdown | An01g09260 | ↑ | Hfd1 |
| Lyso phospholipase, synthesis of glycerophosphocholine | An16g01880 | ↑ |                                  |
| plasma membrane flipase transporting sphingoid long chain bases | An02g06440 | ↑ | Rsb1 |
| glycerophosphocholine phosphodiesterase, synthesis of phosphocholine | An18g03170 | ↑ | Gde1 |
| lanosterol 14A-demethylase | An11g02230 | ↓ | Erg11 |
| C-14 sterol reductase, ergosterol synthesis | An01g07000 | ↓ | Erg24 |
| choline/ethanolamine permease | An01g13290 | ↓ | Hnm1 |
| **Calcium homeostasis and signaling** |                         |         |                                  |
| Ca<sup>2+</sup>/calmodulin dependent protein kinase | An02g05490 | ↓ | Cmk2 |
| **Cell wall remodeling and integrity** |                         |         |                                  |
| endo-glucanase EglA | An14g02760 | ↑ |                                  |
| endo-glucanase EglB | An16g06800 | ↑ |                                  |
| endo-glucanase similar to *Trichoderma reesei* egl4 | An14g02670 | ↑ |                                  |
| alpha-glucan/glucoamylase synthase AfgA (GPI-anchored) | An09g03100 | ↑ |                                  |
| chitin synthase ChsL | An02g02340 | ↑ | Chs3 |
| chitin transglycosidase | An16g02850 | ↑ | Chl1 |
| chitinase | An01g05360 | ↑ | Cts2 |
| cell wall protein similar to *A. nidulans* PhiA | An14g01820 | ↑ |                                  |
| cell wall protein with internal repeats | An12g01200 | ↑ |                                  |
| cell wall protein (flocculin) | An12g00140 | ↑ | Flo11 |
| protein involved in β-1,3 glucan synthesis | An05g00130 | ↓ | Knh1 |
| β-1,2-mannosyltransferase | An04g06730 | ↓ | Mnn2 |
| **Primary metabolism** |                         |         |                                  |
| isocitrate lyase AcuD | An01g09270 | ↑ | Icl1 |
| citrate lyase | An11g00510 | ↑ |                                  |
| citrate lyase | An11g00530 | ↑ |                                  |
| succinate dehydrogenase | An16g07150 | ↑ | Osm1 |
| aspartate transaminase, synthesis of glutamate | An16g05570 | ↑ |                                  |
| acetyl-CoA carboxylase, synthesis of fatty acids | An12g04020 | ↑ |                                  |
| homo-isocitrate dehydrogenase, synthesis of lysine | An15g02490 | ↓ | Lys12 |
| arginosuccinate synthetase, synthesis of arginine | An15g02340 | ↓ | Arg1 |
| acetylornithine aminotransferase, synthesis of arginine | An15g02360 | ↓ | Arg8 |
| arginyl-tRNA synthetase | An02g04880 | ↓ |                                  |
| aspartic beta semi-aldehyde dehydrogenase, synthesis of threonine and methionine | An11g09510 | ↓ | Hom2 |
| homoserine kinase, synthesis of threonine | An17g02090 | ↓ | Thr1 |
| threonine synthase | An16g02520 | ↓ | Thr4 |
| phosphoribosylglycinamidase formyltransferase, synthesis of purines | An02g02700 | ↓ | Ade8 |
| **Secondary metabolism** |                         |         |                                  |
| polyketide synthase | An11g07310 | ↑ |                                  |
| similar to plant zeaxanthin epoxidase ABA2 | An03g06500 | ↑ |                                  |
| similar to enniatin synthase esyn1 of *Fusarium scirpi* | An13g03040 | ↑ |                                  |
| similar to enoyl reductase LovC of the lovastatin biosynthesis *A. terreus* | An13g02940 | ↑ |                                  |
| similar to enoyl reductase LovC of the lovastatin biosynthesis *A. terreus* | An09g01880 | ↑ |                                  |
| similar to HC-toxin peptide synthase HTS of *Cochliobolus carbonum* | An16g06720 | ↑ |                                  |
among the cell wall stress responsive genes when and cell wall integrity in ergosterol metabolism is of main importance for polarized growth to caspofungin or fenpropimorph [45]. This suggests that three strains of the ergosterol synthesizing enzyme Erg24p, is modulated in all is therefore intriguing that expression of An01g07000, the ortholog selective manner to organize signal transduction complexes [63]. It monomeric and trimeric G proteins associate in a dynamic and ordered subdomains of eukaryotic plasma membranes into which control of organize and regulate signaling cascades involved in polar growth concentrate to form lipid rafts in plasma membranes which lipids (e.g. MIPC) and ergosterol are worth highlighting as they function as secondary messengers in eukaryotes (DAG, PA, IP,PIP2, SIP), others are essential components of fungal membranes (plasma membrane, organelles, lipid droplets), whereby sphingolipids (e.g. MIPC) and ergosterol are worth highlighting as they concentrate to form lipid rafts in plasma membranes which organize and regulate signaling cascades involved in polar growth control of S. cerevisiae [62]. Lipid rafts have been shown to form ordered subdomains of eukaryotic plasma membranes into which monomeric and trimeric G proteins associate in a dynamic and selective manner to organize signal transduction complexes [63]. It is therefore intriguing that expression of An01g07000, the ortholog of the ergosterol synthesizing enzyme Erg24p, is modulated in all three strains ∆racA, ramona-1 and PygaA-racAT, and being also among the cell wall stress responsive genes when A. niger is exposed to caspofungin or fenpropimorph [45]. This suggests that ergosterol metabolism is of main importance for polarized growth and cell wall integrity in A. niger.

Unfortunately, data on fungal lipid signaling networks are sparse. So far, it is known that sphingolipids play a key role in pathogenicity in Cryptococcus neoformans, that the quorum sensing molecule farnesol is involved in mycelial growth, biofilm formation and stress response of Candida albicans, that both sphingolipids and farnesol are important for maintaining cell wall integrity and virulence of A. fumigatus (for review see [59]) and that the activity of two ceramide synthases is important for the formation of a stable polarity axis in A. nidulans [64] (Li et al.). In Schizosaccharomyces pombe, MIPC was shown to be required for endocytosis of a plasma-membrane-localized transporter and for protein sorting into the vacuole [65]. As ∆racA is affected in endocytosis (see below) and the MIPC synthesizing enzyme Sur1p (An05g02310) is down-regulated as well might suggest that MIPC has a similar function in A. niger. Notably, the sphingolipid synthesizing protein inositol-phosphoryl ceramide synthase (ipc1) plays a major role in both establishment and maintenance of cell polarity in A. nidulans

| Table 4. Cont. |
|----------------|
| **Predicted protein function** | **Open reading frame code** | **Up/Down** | **Closest S. cerevisiae ortholog** |
| Transporter | polyamine transporter | An11g07300 | ↑ | Tpo3 |
| polyamine transporter | An12g07400 | ↑ | Tpo3 |
| polyamine transporter | An13g03220 | ↓ | Tpo1 |
| vacuolar basic amino acid transporter | An06g00770 | ↑ | Vba5 |
| oligopeptide transporter | An11g01040 | ↓ | Opt1 |
| hexose transporter | An02g07610 | ↑ | Hxt5 |
| galactose transporter | An01g10970 | ↓ | Gal2 |
| low-affinity Fe(II) transporter of the plasma membrane | An16g06300 | ↓ | Fet4 |
| siderophore transporter | An03g03560 | ↓ | Arn1 |
| plasma membrane multidrug transporter | An07g01250 | ↓ | Pdr5 |
| multidrug transporter | An13g03060 | ↑ | Snp2 |
| **Protein trafficking** | | | |
| protein kinase involved in exocytosis | An08g03360 | ↑ | Kin1 |
| **Other signaling processes** | | | |
| zinc finger transcriptional repressor | An04g08620 | ↑ | Oaf3 |
| protein recruiting the SAGA complex to promoters | An07g04540 | ↑ | Ctb6 |
| histidine kinase osmosensor | An14g02970 | ↑ | Sin1 |
| transcriptional regulator involved in nitrogen repression | An02g11830 | ↑ | Ure2 |
| transcription factor similar to A. nidulans MedA | An02g02150 | ↑ | |
| transcription factor | An02g06180 | ↑ | |
| transcription factor important for salt stress resistance | An12g09020 | ↑ | Hal9 |
| DNA damage checkpoint protein during replication | An03g06930 | ↓ | Rad24 |
| SUN family protein involved in replication | An08g07090 | ↓ | Sim1 |
| transcription factor important for Zn²⁺ homeostasis | An08g01860 | ↓ | Zap1 |
| alpha subunit of the translation initiation factor | An18g04650 | ↓ | Gcn3 |
| **Others** | | | |
| pathogenesis-related protein | An08g05010 | ↑ | Pry1 |
| hypothetical aspergillosis allergen rAsp | An03g00770 | ↓ | |
Table 5. GO term enriched actin-related genes whose expression responds to the switch from polar to apolar growth in *PylaA*-raca<sup>Δ78V</sup>.

| Predicted protein function* | Open reading frame code | Up/Down | Closest *S. cerevisiae* ortholog |
|----------------------------|-------------------------|---------|-------------------------------|
| Rho GDP dissociation inhibitor | An18g04590 | ↑ | Rdi1 |
| TORC2 and phosphoinositide PI4,5P(2) binding protein | An11g02840 | ↑ | Sim2 |
| Arp2/3 complex subunit | An02g06360 | ↑ | Arc15 |
| Arp2/3 complex subunit | An01g05510 | ↑ | Arc35 |
| tropomyosin 1 | An18g06590 | ↑ | Arc40 |
| actin cortical patch component | An13g00760 | ↑ | Tpm1 |
| actin-capping protein | An02g14620 | ↑ | Aip1 |
| protein recruiting actin polymerization machinery | An10g00370 | ↑ | Bzz1 |

Genes up-regulated are indicated with ↑. Differential gene expression was evaluated by moderated t-statistics using the Limma package [82] with a FDR threshold at 0.05 [83]. Fold changes and statistical significance is given in Additional file 1 and 2.

* Protein functions were predicted based on information inferred from the Saccharomyces genome data base SGD (http://www.yeastgenome.org/) and the Aspergillus genome database AspGD (http://www.aspergillusgenome.org/).

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by regulating actin dynamics [66,67]. However, it is not known whether this is mediated by the sphingolipid moieties of phospholipid ceramide (IPC) or by other products of the ceramide synthetic pathway such as DAG, MIPC or sphingosines. Anyhow, inhibition of sphingolipid synthesis in *A. nidulans* caused wider hyphal cells, abnormal branching and tip splitting and is not suppressible by the addition of sorbitol [66,67] - observations which also hold true for ΔracA and ramosa-1 [24,25], suggesting that sphingolipid mediated control of hyphal cell polarity is not mediated by the CWI pathway in *Aspergillus*. Still, *S. cerevisiae* strains defective in CWI signaling (e.g. pka1Δ, mpk1Δ) also exhibit severe defects in lipid metabolism, including accumulation of phosphatidylcholine, DAG, triacylglycerol, and free sterols as well as aberrant turnover of phosphatidylcholine, suggesting that CWI signaling and lipid homeostasis are nevertheless closely linked in fungi [68].

A second important outcome of this study is that not only calcium signaling seems to be of utmost importance for morphological decisions in all three mutant strains ΔracA, ramosa-1 and *PylaA*-raca<sup>Δ78V</sup>, but ion homeostasis in general (Fig. 7). Many ion transport proteins are differentially expressed in all three strains when compared to the wild-type situation including transport proteins for Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Fe<sup>2+</sup>, Zn<sup>2+</sup> and Co<sup>2+</sup>. Of special importance is An16g06300, a predicted Fe(II) transporter, homologous to the *S. cerevisiae* plasma membrane transporter Fet4p, whose transcriptional regulation is affected in all three strains. Fet4p is a low-affinity Fe(II) transporter also transporting Zn<sup>2+</sup> and Co<sup>2+</sup> and is under combinatorial control of iron (Atf1p transcriptional activator), zinc (Zap1p transcriptional factor) and oxygen (Rox1p repressor) [69], being for example important for *S. cerevisiae* to tolerate alkaline pH [70]. It has been postulated that changes in the phospholipid composition govern the function of membrane-associated zinc transporters such as Fet4p [71]. Vice versa, the transcriptional factor Zap1p controls not only expression of zinc-related transporters but also expression of the DAG pyrophosphate phosphatase Dppp1p [71]. This is a very interesting observation in view of the fact that one predicted Dppp1p ortholog (An01g03870) shows differential expression in all three morphological mutant strains of *A. niger*, an analogy which might suggest that polarized growth of *A. niger* might be orchestrated by (phospho)lipid signaling which is somehow interconnected with zinc metabolism.

Finally, our transcriptomics comparison uncovered that endocytotic processes are likely to be involved in the morphogenetic network of *A. niger*. In all three strains, ΔracA, ramosa-1 and *PylaA*-raca<sup>Δ78V</sup>, expression of An03g01160, a predicted ortholog of the *S. cerevisiae* Lsb4p was modulated (Table 6, Fig. 7). Lsb4p is an actin-binding protein, conserved from yeast to humans, binds to actin patches and promotes actin polymerization together with the WASP protein Lsb17 in an Arp2/3-independent pathway thereby mediating inward movement of vesicles during endocytosis [72]. Lsb4p is also a PIP binding protein due to a phosphoinositide-binding domain (SYLF), which is highly conserved from bacteria to humans. The human homolog of Lsb4p (SH3YL1) binds to PIP3 and couples the synthesis of PIP2 with endocytotic membrane remodeling, whereas Lsb4p binds directly to PIP2 (note that PIP3 is believed to be absent in yeast). Thus, Lsb4p homologs are predicted to couple PIP2 with actin polymerization to regulate actin and membrane dynamics involved in membrane ruffling during endocytosis [73]. Beside An03g01160 (Lsb4p ortholog), An17g01945 is worth highlighting in this context as well. An17g01945 encodes an ortholog of the amphipath-like lipid raft protein Rvs161p and is differentially expressed in both ΔracA and *PylaA*-raca<sup>Δ78V</sup>. Rvs161p affects the membrane curvature in *S. cerevisiae* and mediates in conjunction with Rvs167 and PIP2 membrane scission at sites of endocytosis [74].

Taken together, the transcriptomic signature of the three morphological mutants predicts that the morphological changes are brought about the interconnection of several signaling and metabolic pathways. Remarkably, the responding gene set in ΔracA and ramosa-1 seems to be, although substantially overlapping, oppositely regulated. One explanation might be that inactivation of RacA and TORC2 induces dichotomous branching in different manners. As the subcellular distribution of actin is different in both strains (the ramosa-1 mutant shows scattered actin patches at hyphal tips, whereas actin is concentrated at hyphal apices in the ΔracA mutant) might suggest that different causes (loss of actin polarization/actin hyperpolarization) provoke different responding transcriptional changes, which, however, eventually result in the same phenotypic response, namely tip splitting.
Table 6. Complete list of genes whose expression respond to hyperbranching in ΔracA versus wild-type and to the switch from polar and apolar growth in PglaA-racAG18V versus PglaA-racA (4 h after induction).

| Predicted protein function* | Open reading frame code | Up/Down in PglaA-racAG18V versus PglaA-racA (4 h) | Up/Down in ΔracA versus wt | Closest S. cerevisiae ortholog |
|-----------------------------|-------------------------|--------------------------------------------------|-----------------------------|-------------------------------|
| **(Phospho)lipid metabolism and signaling** |
| phospholipase B, synthesis of glycerophosphocholine | An18g01090 | ↓ | ↓ | Plb3 |
| diacylglycerol pyrophosphate phosphatase, synthesis of DAG | An02g01180 | ↓ | ↓ | Dpp1 |
| diacylglycerol pyrophosphate phosphatase, synthesis of DAG | An04g03870 | † | † | Dpp1 |
| sterol 24-C-methyltransferase, ergosterol synthesis | An04g04210 | † | † | Erg6 |
| C-14 sterol reductase, ergosterol synthesis | An01g07000 | ↓ | ↓ | Erg24 |
| transcription factor important for sterol uptake | An02g07950 | ↓ | ↓ | Upc2 |
| transcription factor important for sterol uptake | An12g00680 | ↓ | ↓ | Upc2 |
| **Cell wall remodeling and integrity** |
| α-1,3-mannosyltransferase | An15g04810 | ↓ | ↓ | Mnt2 |
| endo-mannanase (GPI-anchored), DfgE | An16g08090 | ↓ | ↓ | Dfg1 |
| β-1,4-glucanase | An03g05530 | ↓ | ↓ | Sps22 |
| cell wall protein | An11g01190 | ↓ | ↓ | Sps22 |
| plasma membrane protein | An02g08030 | ↓ | ↓ | Pmp3 |
| **Actin localization** |
| aminophysin-like protein required for actin polarization | An17g01945 | ↓ | ↓ | Rvs161 |
| actin-binding protein involved in endocytosis | An03g01160 | ↓ | ↓ | Lsb4 |
| **Transporter** |
| choline/ethanolamine permease | An01g13290 | ↓ | ↓ | Hnn1 |
| low-affinity Fe(II) transporter | An16g06300 | ↓ | ↓ | Fet4 |
| oligopeptide transporter | An11g01040 | ↓ | ↓ | Opt1 |
| galactose/glucose permease | An01g0970 | ↓ | ↓ | Gal2 |
| **Protein trafficking** |
| GTPase activating protein involved in protein trafficking | An01g02860 | ↓ | ↓ | Gyp8 |
| protein important for endosomal-vacuolar trafficking | An11g01810 | ↓ | ↓ | Rcr2 |
| peptidase | An03g02530 | ↓ | ↓ | |
| **Others** |
| hypothetical aspergillosis allergen rAsp | An03g00770 | ↓ | ↓ | |
| cytochrome P450 protein | An11g02990 | ↓ | ↓ | Dit2 |
| isoamyl alcohol oxidase | An03g06270 | ↓ | ↓ | |
| protein with strong similarity to penicillin V amidohydrolase | An12g04630 | ↓ | ↓ | |
| oxidoreductase | An03g00280 | ↑ | ↑ | |
| protein with nucleotide binding domain | An01g08150 | ↑ | ↑ | Ire24 |
| protein with methyltransferase domain | An09g00160 | ↑ | ↑ | |
| protein with unknown function | An15g03880 | ↓ | ↓ | |
| protein with unknown function | An01g10900 | ↓ | ↓ | |
| protein with unknown function | An07g05820 | ↓ | ↓ | |
| protein with unknown function | An18g00810 | ↓ | ↓ | |
| protein with unknown function | An04g04630 | ↓ | ↓ | |
| protein with unknown function | An06g00320 | ↓ | ↓ | |
| protein with unknown function | An07g04900 | ↓ | ↓ | |
| protein with unknown function | An01g13320 | ↓ | ↓ | |
| protein with unknown function | An16g07920 | ↑ | ↑ | |
| protein with unknown function | An01g03780 | ↓ | ↓ | |

Genes up-regulated are indicated with ↑, genes down-regulated with ↓. Differential gene expression was evaluated by moderated t-statistics using the Limma package (82) with a FDR threshold at 0.05 (83). Identical ORFs or proteins with predicted similar function being also differentially expressed in ramosa-1 are indicated in bold. Fold changes and statistical significance is given in Additional file 1 and 2.

*: Protein functions were predicted based on information inferred from the Saccharomyces genome data base SGD (http://www.yeastgenome.org/) and the Aspergillus genome database AspGD (http://www.aspergillusgenome.org/).

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Similarly puzzling is the observation that the core set of 38 genes which are responsive in both $D_{racA}$ and $P_{glaA-racAG18V}$ respond in the same direction (Table 6), although they are associated with excessive polar growth (hyperbranching) in the $D_{racA}$ strain but with the absence of polar growth (tip swelling) in strain $P_{glaA-racAG18V}$. A plausible explanation might be that loss of polarity maintenance in both strains is connected with similar transcriptional changes controlling actin dynamics and vesicle flow, but that reestablishment of polar growth in the $D_{racA}$ deletion strain requires genes which are not important for tip swelling in the $P_{glaA-racAG18V}$ strain.

Does a hyperbranching strain secrete more proteins?

In filamentous fungi, it is believed that protein secretion occurs at the hyphal tip. This holds true for glucoamylase (GLA), the most abundant secreted enzyme in $A. niger$ [11,12]. Another example is z-amylase, the major secretory protein of $A. oryzae$ [14]. Hence, one might expect that a higher branching frequency in $D_{racA}$ strain would result in higher secretion yields. However, our study demonstrated that more tips in the $D_{racA}$ strain do not necessarily increase protein secretion; instead, protein yields were the same in both mutant and wild-type (Table 2). The most logical explanation is that the same amount of secretory vesicles is merely distributed to more tips in the $D_{racA}$ strain, resulting in less secretory vesicles per individual tip. The quantitative data obtained for the exocytotic marker GFP-SncA and the endocytotic marker AbpA-CFP and SlaB-YFP clearly demonstrate that fewer vesicles are transported to the apex of an individual tip (Fig. 3) and that endocytosis is slowed down as well – the endocytotic ring seems to be less well defined and the fluorescence intensity of both endocytotic markers is decreased (Fig. 4). This data is corroborated by the transcriptomic fingerprint of the $D_{racA}$ strain. The transcription of genes predicted to function in protein trafficking and actin localization is downregulated as well as expression of genes governing phospholipid signaling and cell wall remodeling. Remarkably, biomass formation is the same in both $D_{racA}$ and wt. This suggests that the amount of secreted vesicles is adjusted in both strains just to ensure hyphal tip growth but that the capacity of a hyphal tip growing apparatus to accommodate vesicles is much higher (at least in $D_{racA}$). Hence, challenging the $D_{racA}$ strain to overexpress a certain protein of interest might increase the number of secretory vesicles thus resulting in higher secretion yields. We currently run respective experiments to test this hypothesis. In any case, the hyperbranching $D_{racA}$ mutant could already be of value for high-density cultivation during industrial processes: it forms a less shear stress-sensitive, compact macromorphology but does not form

Figure 7. A reconstructed model for the morphogenetic network of $A. niger$ based on the transcriptomic fingerprints determined for the apical branching mutant $ramosa-1$ [25], the apical branching mutant $\Delta{\text{rac}}$ (this work) and the apolar growing mutant $P_{glaA-RacAG18V}$ (this work). The model also rests on cell biological and phenotypic data obtained for all three strains (this work and [25]) as well as on literature data for conserved mechanisms from yeast to humans (see discussion for references). Indicated are signalling and metabolic processes, which showed transcriptional responses in all three strains, some deduced key players and the hypothetical connection of these processes. doi:10.1371/journal.pone.0068946.g007
pellets. It thus exhibits improved rheological properties without any apparent disadvantages with respect to growth rate and physiology.

**Conclusions**

The transcriptomic signature of the three individual mutants $\Delta racA$, *ramosa-1* and *Pg-lac-racA* uncovered specific and overlapping responses to the morphological changes induced and suggests the participation as well as interconnectedness of several regulatory and metabolic pathways in these processes. The data obtained predict a role for different signaling pathways including phospholipid signaling, sphingolipid signaling, TORC2 signaling, calcium signaling and CWI signaling in the morphogenetic network of *A. niger*. These pathways likely induce different physiological adaptations including changes in sterol, zinc and amino acid metabolism and changes in ion transport and protein trafficking. Central to the morphological flexibility of *A. niger* is the actin cytoskeleton whose dynamics can be precisely controlled in these mutants. Future attempts are necessary to address important issues which cannot be resolved by transcriptomics. For example, how is the lipid composition in apical and subapical regions of *A. niger* hyphae? Which morphogenetic proteins are also parts of the network whose expression is regulated post-transcriptionally and can thus not be detected by transcriptomics approaches? Where are they localized during (a)polar growth? What are the metabolic prerequisites to sustain fast polar growth coupled with high secretion rates? Clearly, a comprehensive understanding of the morphogenetic network of *A. niger* will require and integrated systems biology approach where transcriptomics analyses will be combined with proteomics, metabolomics and lipidomics approaches and linked with cell biological studies.

**Materials and Methods**

**Strains, culture conditions and molecular techniques**

*Aspergillus* strains used in this study are given in Table 7. Strains were grown on minimal medium (MM) [75] containing 1% (w v$^{-1}$) glucose and 0.1% (w v$^{-1}$) casamino acids or on complete medium (CM), containing 0.5% (w v$^{-1}$) yeast extract in addition to MM. When required, plates were supplemented with uridine (10 mM). Transformation of *A. niger* and fungal chromosomal DNA isolation was performed as described [76]. All molecular techniques were carried out as described earlier [77].

**Bioreactor cultivation conditions**

Maltose-limited batch cultivation was initiated by inoculation of 5 L (kg) ammonium based minimal medium with conidial suspension to give $10^{9}$ conidia L$^{-1}$. Maltose was sterilized separately from the MM and final concentration was 0.8% (w/v). Temperature of 30°C and pH 3 were kept constant, the latter by computer controlled addition of 2 M NaOH or 1 M HCl, respectively. Acidification of the culture broth was used as an indirect growth measurement [78]. Submerged cultivation was performed with 6.6 L BioFlo3000 bioreactors (New Brunswick Scientific, NJ, USA). A more detailed description of the fermentation medium and cultivation is given in [79]. Batch cultivation for *Pg-lac-racAG18V* or *Pg-lac-RacA* were run similarly as the maltose-limited batch cultivations of $\Delta racA$ cultures except that 0.75% xylose was used as a initial carbon source instead of maltose. When the exponential growth phase was over (indicated by a sharp rise of the dissolved oxygen tension and the pH value), 0.75% maltose was added to induce expression of *Pg-lac-racAG18V* or *Pg-lac-RacA*, respectively. Samples for the analysis of morphological characteristics, biomass formation, protein yield and RNA were taken every hour.

**Analysis of culture broth**

Dry weight biomass concentration was determined by weighing lyophilized mycelium separated from a known mass of culture broth. Culture broth was filtered through GF/C glass microfiber filters (Whatman). The filtrate was collected and frozen for use in solute analyses. The mycelium was washed with demineralised water, rapidly frozen in liquid nitrogen and lyophilized. Glucose concentration was measured as previously described [80] with slight modifications: 250 mM triethanolamine (TEA) was used as buffer (pH 7.5). Extracellular protein concentration was determined using the Quick Start Bradford Protein Assay (Bio-Rad) using BSA as standard. The total organic carbon in the culture filtrate was measured with a Total Organic Carbon Analyzer (TOC-Vcsn; Shimadzu, Japan) using glucose as standard.

**Microarray analysis**

Total RNA extraction, RNA quality control, labeling, Affymetrix microarray chip hybridization and scanning were performed as previously described [25]. Background correction, normalization and probe summarization steps were performed according to the default setting of the robust multi-array analysis (RMA) package as recently described [81]. Differential gene expression was evaluated by moderated t-statistics using the Limma package [82] with a threshold of the Benjamini and Hochberg False Discovery Rate (FDR) of 0.05 [83]. Fold change of gene expression from different samples was calculated from normalized expression values. Geometric means of the expression values as well as fold change for all strains and comparisons are summarized in Tables S1 and S2 and have been deposited at the GEO repository (http://www.ncbi.nlm.nih.gov/geo/info/linking.html) under the accession number GSE42258. Transcriptomic data for the exponential growth phase of the reference strain N402 was published recently [94].

**Gene Ontology (GO) and enrichment analysis**

Over-represented GO terms in sets of differentially expressed genes were determined by Fisher’s exact test [85] as implemented in FetGOat [43] using a FDR of q<0.05. An improved GO
amplification for \textit{A. niger} CBS513.88 was applied based on ontology mappings from \textit{Aspergillus} FGSCA4 [43].

Construction of AbpA-CFP and SlaB-YFP expression cassettes

Standard PCR and cloning procedures were used for the generation of the constructs [77]. All PCR amplified DNA sequences and cloned fragments were confirmed by DNA sequencing (Macrogen). Primers used in this study are listed in Table S5. Correct integrations of constructs in \textit{A. niger} were verified by Southern analysis [77]. The expression vectors, AbpA-CFP and SlaB-YFP were constructed using the fusion PCR approach as described previously [23] with slight modifications. Plasmid pVM3-1 [23] harboring the GA5 peptide linker followed by the CFP, TmpC, and the selection marker \textit{pyrG} from \textit{A. oryzae} was used as starting point. A second TmpC terminator sequence was generated by PCR and ligated via a SfiI restriction site into pVM3-1 which would later allow looping out of the \textit{pyrG} marker by FQA counter-selection [76]. The resulting plasmid was named pMK3. For the fusion PCR, three separate fragments were amplified by PCR: the C-terminal part of \textit{abpA} ORF (~1 kb), the module containing CFP-TmpC-AopyrG (~3.5 kb) and the terminator region of \textit{abpA} (~1 kb). Subsequently, the three individual fragments were fused together by a fusion PCR and the resulting amplicon (~5.6 kb) was cloned into pJET (Fermentas) to give plasmid pMK5. SlaB-YFP was also constructed in a similar way and the final plasmid was named pMK6.

Microscopy

Light microscopic pictures were captured using an Axiosplan 2 (Zeiss) equipped with a DKC-5000 digital camera (Sony). For light and fluorescence images for SlaB-YFP and AbpA-CFP transformants, pictures were captured with 40× C-apolachromatic objective on an inverted LSM5 microscope equipped with a laser scanning confocal system (Zeiss Observer). The observation conditions for the life-imaging of hyphae were described previously [24]. To determine branching frequencies, the lengths of hyphae and branches were measured and evaluated using the program Image J. For the quantification of GFP-SncA, AbpA-CFP and SlaB-YFP signals, a single section of individual hyphal tip was captured (n>20).

Supporting Information

Table S1 Complete transcriptome data containing RMA expression values (log2 scale), mean expression values, p-values, q-values and fold changes.

Table S2 Subset of the transcriptome data containing up-down-regulated gene sets.

Table S3 ZIP archive file containing FetGOat enrichment results for the up- and down-regulated gene sets of all six comparisons.

Table S4 Subset of the transcriptome data for selected intersection of the Venn diagram.

Table S5 Primers used in this study.

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Author Contributions

Conceived and designed the experiments: MJK TRJ AFJR VM. Performed the experiments: MJK BMN MA TRJ. Analyzed the data: MJK BMN TRJ VM. Contributed reagents/materials/analysis tools: BMN AFJR VM. Wrote the paper: MJK AFJR VM.
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