Refining the pH response in Aspergillus nidulans: a modulatory triad involving PacX, a novel zinc binuclear cluster protein

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Summary

The Aspergillus nidulans PacC transcription factor mediates gene regulation in response to alkaline ambient pH which, signalled by the Pal pathway, results in the processing of PacC72 to PacC27 via PacC53. Here we investigate two levels at which the pH regulatory system is transcriptionally moderated by pH and identify and characterise a new component of the pH regulatory machinery, PacX. Transcript level analysis and overexpression studies demonstrate that repression of acid-expressed palF, specifying the Pal pathway arrestin, probably by PacC27 and/or PacC53, prevents an escalating alkaline pH response. Transcript analyses using a reporter and constitutively expressed pacC trans-alleles show that pacC preferential alkaline-expression results from derepression by depletion of the acid-prevalent PacC72 form. We additionally show that pacC repression requires PacX. pacX mutations suppress PacC processing recalcitrant mutations, in part, through derepressed PacC levels resulting in traces of PacC27 formed by pH-independent proteolysis. pacX was cloned by impala transposon mutagenesis. PacX, with homologues within the Leotiomyceta, has an unusual structure with an amino-terminal coiled-coil and a carboxy-terminal zinc binuclear cluster. pacX mutations indicate the importance of these regions. One mutation, an unprecedented finding in A. nidulans genetics, resulted from an insertion of an endogenous Fot1-like transposon.

Introduction

Fungi, ubiquitous in nature, occupy niches of wide ranging and fluctuating pH values. This is enabled by efficient pH homeostasis and a pH regulatory system that ensures the appropriate synthesis of molecules with respect to environmental pH. The system mediating this response was first recognised in Aspergillus nidulans (Caddick et al., 1986). Homologous systems occur throughout the ascomycetes where they are known as the Pac/Pal system in the filamentous fungi (Caddick et al., 1986; Tilburn et al., 1995) and as the Rim system in yeasts (Su and Mitchell, 1993a,b; Lambert et al., 1997; Ramon et al., 1999; Davis et al., 2000a,b). They also extend to the basidiomycetes (Aréchiga-Carvajal and Ruiz-Herrera, 2005; O’Meara et al., 2010; Ost et al., 2015).

The fungal pH responsive regulatory domain encompasses a very large number of genes including those involved in nutrient acquisition, ion homeostasis, alkaline metal and pH tolerance, cell wall metabolism, exported metabolite production, female development, sporulation, dimorphic shift, tissue penetration and invasive growth (Lamb et al., 2001; Lamb and Mitchell, 2003; Bensen et al., 2004; Eisendle et al., 2004; Baek et al., 2006; Ruiz and Ariño, 2007; Nobile et al., 2008; Alkan et al., 2013; Trushina et al., 2013; Bertuzzi et al., 2014; Chinnici et al., 2014; O’Meara et al., 2014). As many of these activities or attributes are crucial in a host environment, pH regulation is an important virulence determinant of fungal pathogenicity of animals, including humans, plants and fungi them-
selves (Davis et al., 2000a; Davis, 2003; Bignell et al., 2005; Moreno-Mateos et al., 2007; Nobile et al., 2008; Zou et al., 2010; Alkan et al., 2013; Trushina et al., 2013; Bertuzzi et al., 2014; O’Meara et al., 2014) and reviewed in Peñalva et al. (2008), Davis (2009), Selvig and Alspaugh (2011) and Cornet and Gaillardin (2014).

The mechanism of pH regulation has been studied largely in A. nidulans and Saccharomyces cerevisiae with additional contributions particularly from work in Candida albicans and Yarrowia lipolytica. The pH response is mediated by the three Cys2-His2 finger transcription factor, A. nidulans PacC (Tilburn et al., 1995) or Rim101 in S. cerevisiae (Su and Mitchell, 1993b). Under acidic conditions, the A. nidulans PacC full-length form, PacC\textsuperscript{72}, is protease inaccessible due to intramolecular-interactions involving the C-terminal moiety (Espeso et al., 2000). At neutral to alkaline ambient pH PacC undergoes two-step proteolysis. The first step, which occurs in response to pH signalling, removes approximately 180 C-terminal residues to yield PacC\textsuperscript{27} (Díez et al., 2002); the second, almost certainly mediated by the proteasome, removes a further ∼240 residues to give the PacC\textsuperscript{27} processed form and is pH-independent (Hervás-Aguilar et al., 2007). PacC\textsuperscript{27} predominates in the nucleus (Mingot et al., 2001), where it activates alkaline-expressed genes, such as ipnA (isopenicillin-N synthase) (Espeso and Peñalva, 1996) and represses acid-expressed genes (Tilburn et al., 1995), such as gabA (GABA permease) (Espeso and Arst, 2000). However, PacC\textsuperscript{72} and PacC\textsuperscript{53} can bind a PacC DNA target site (Díez et al., 2002) and, as they are not excluded from the nucleus (Mingot et al., 2001; Davis, 2003; Fernández-Martínez, A Hervás-Aguilar MAP, EAE, unpublished), might also participate in gene regulation.

pacC mutations that remove the PacC\textsuperscript{72} C-terminus or otherwise disrupt its intramolecular-interactions result in an open, proteasome-accessible conformation leading to constitutive PacC processing and alkalinity mimicry (Orejas et al., 1995; Tilburn et al., 1995; Espeso et al., 2000). Loss-of-function mutations in the pal (pH signal transduction) genes or pacC result in acidity mimicry (Arst et al., 1994; Tilburn et al., 1995; Fernández-Martínez et al., 2003). It appears that traces of PacC\textsuperscript{72} are produced independently of pH signal transduction from a minor proportion of protease accessible PacC\textsuperscript{72} that exists in equilibrium with the majority of protease inaccessible PacC\textsuperscript{72} (Peñalva and Arst, 2004; Peñas et al., 2007; Peñalva et al., 2008). This possibly explains the less extreme phenotype of null pal compared with null pacC mutations, which result, additionally, in cryosensitivity and reduced growth and conidiation (Tilburn et al., 1995; Fernández-Martínez et al., 2003).

pH signalling occurs at the plasma membrane in A. nidulans (Galindo et al., 2012; Lucena-Agell et al., 2015) and S. cerevisiae (Obara and Kihara, 2014) where it involves dedicated Pal (Arst et al., 1994) (or Rim) pH signal transduction components and the participation of certain endosomal sorting complex required for transport (ESCRT)-I, -II and -III components (Xu et al., 2004; Calcagno-Pizarelli et al., 2011; reviewed by Peñalva et al., 2014). The plasma membrane sensor is PalH [Rim21 and Dgf16 in S. cerevisiae and C. albicans (Barwell et al., 2005; Rothfels et al., 2005)]. PalH localisation is assisted by PalC (Calcagno-Pizarelli et al., 2007) and stabilised by strong interactions between the PalH cytoplasmic tail and the arrestin PalF (Herranz et al., 2005) (Rim8). PalF becomes phosphorylated and ubiquitylated in alkaline media (Herranz et al., 2005), and the importance of this ubiquitylation, in A. nidulans, is demonstrated by the ability of genetically encoded ubiquitin attachment to PalF to signal constitutively (Hervás-Aguilar et al., 2010). PalF recruits Vps23 of ESCRT-I (Herrador et al., 2010; Galindo et al., 2012), which is thought to recruit ESCRTII components. Vps32 of ESCRT-III participates in the incorporation of PalC (Galindo et al., 2007) [probably YGR122w in yeast and named YiRim23 in Y. lipolytica (Blanchin-Roland, 2011)], which is required for inclusion of PalA (Galindo et al., 2012) (Rim20). PalA binds PacC\textsuperscript{27} via two YPxl(I) motifs flanking the signalling protease (PalB) cleavage site (Vincent et al., 2003). Finally the cysteine protease PalB (Denison et al., 1995) (Rim13) is recruited through interaction of its MIT domain with Vps24 (Rodríguez-Galán et al., 2009; Lucena-Agell et al., 2015). The transient signalling foci are dissociated by Vps4 (Galindo et al., 2012).

Mutations in pacC that prevent PacC signalling proteolysis affect the signalling proteolysis site (Díez et al., 2002; Peñas et al., 2007) or PalA binding sites (Vincent et al., 2003) and are phenotypically identical to those in the pal signal transduction genes except that, unlike pal mutations that are recessive, they are co-dominant with the wild-type allele in diploids (see below).

The Pac/Pal system mediates a rapid and effective response to alkalisation, switching genes on or off as appropriate to ensure survival in these adverse conditions. However, fungi also grow in acidic environments and normally prefer to do so. A. nidulans can grow in media of pH values as low as pH 2 (Dijkema et al., 1986) and in a study of the influence of pH on the growth of toxigenic Aspergillus, Penicillium and Fusarium species, the majority of the 61 isolates were able to grow around pH 3–3.5 and some as low as pH ∼ 2 (Wheeler et al., 1991). To adapt to acidic conditions, fungi must be able to control, even switch off, the alkaline ambient pH response. Here we describe, for A. nidulans, a tripartite system whereby this is achieved that includes a new player in the pH response.
**Results**

New insights into the pacC/pal pH regulatory response on pacC autoregulation, revisited. pacC is an alkaline expressed gene. In wild-type strains pacC transcript levels are low under acidic conditions and relatively high under alkaline conditions (Tilburn et al., 1995). In addition, they are low in acidity mimicking pal strains and certain pacC/pal partial loss-of-function strains and relatively high in pacC constitutive, alkalinity mimicking mutants, irrespective of the growth pH (Tilburn et al., 1995). This strongly suggested that pacC is positively regulated by PacC27, in a similar manner to alkaline expressed structural genes, such as ipnA (Orejas et al., 1995; Tilburn et al., 1995; Espeso and Peñalva, 1996).

However, the hypothesis of pacC positive autoregulation failed to explain a number of subsequently observed phenomena. First, in contrast to pal alleles that are recessive to the respective wild-type alleles in diploids, pacC processing recalcitrant alleles pacC−/−205, pacC−/−207, pacC−/−209 and pacC−/−210 are co-dominant with the wild-type allele in diploids, a feature that enabled the isolation of pacC−/−207, pacC−/−209 and pacC−/−210 as suppressors for GABA utilisation in a homozygous areA (unable to use nitrogen sources other than ammonium) diploid (Arst et al., 1994; Espeso and Arst, 2000; Diez et al., 2002; Fernández-Martínez et al., 2003; Vincent et al., 2003).

Second, and in agreement, there are reduced levels of expression of pacC700, a GFP tagged allele encoding PacC27, in a diploid containing pacC−/−209 as compared with that of a pacC700 haploid strain or of pacC700 in a diploid strain containing a pacC null allele, as detected by epifluorescence microscopy (Fig. S1A). Third, there exists an apparently paradoxical phenomenon whereby the hypostasis of a pal allele and the epistasis of a pacC allele can be inverted by ectopic overexpression of pacC from a strong heterologous [a/a (alcohol dehydrogenase)] promoter in a pacC pal strain (Fig. S1B and J, M Mingot, M Orejas, T Suárez, EAE, MAP, HNA, unpublished). All of these observations indicate that there is a negative function or activity associated with the PacC unprocessed form.

The testing of the original hypothesis directly was enabled by the isolation of the pacC6309 null allele, which differs from the wild-type transcript in three nucleotides and contains a chain termination mutation in the physiological start codon, such that it can specify at most PacC residues 1 through 4 by using an alternative start codon (Fernández-Martínez et al., 2003). The northern blots in Fig. 1A show the results of pH shift experiments. In the wild-type strain pacC transcript levels are low under acidic conditions and rapidly rise upon alkalisation to peak approximately 15 min after shifting, falling to steady-state levels after about 60 min. In contrast, in the pacC6309 null strain, pacC transcript levels are largely constant throughout and considerably elevated relative to those of the wild-type strain under acidic conditions. As PacC72 is the almost exclusive PacC form in acidic media, this strongly implicates PacC72 as a repressor of pacC.
Western blots illustrating PacC processing. (A) pH signalling proteolysis occurs rapidly after alkalisation and (B) becomes rate limiting in PacC processing after 1.5–2 h. (A) and (B) Mycelia were grown in acidic media and transferred to alkaline media for the times shown. A pacC900 [MYC3-PacC, (Peñas et al., 2007)] strain MAD2352 wA4 pyroA4 inoB2 palF500 pyrG89 nkuA::::bar pacC900 was used. [palF500 is palF::HA3::pyrGfum, a palF tagged allele] (Hervás-Aguilar et al., 2010).

To investigate the possibility of PacC72 repressor function further we used pacC 6309 transcript as a reporter for pacC expression in the presence and absence of different pacC alleles in trans, expressed from a moderately strong constitutive promoter. Fig. 1B shows that pacC 6309 transcript levels (lane 2) are unaffected by the expression of trans-acting in diploids with wild-type or constitutive alleles because their gene product represses the heteroallelic promoter (Fig. S1A). In a similar way, in a haploid, a pal allele becomes epistatic to a pacC allele when pacC is ectopically overexpressed due to large amounts of PacC72 switching off expression of the pal-independent pacC allele (Fig. S1B).

Transcriptional regulation of palF prevents a run-away alkaline pH response. Figure 2 illustrates PacC processing. In response to alkalisation, PacC72 is processed via PacC53 to PacC27. The response is very swift with PacC72 appearing after 4 min (Panel A) and, in fact, as early as 2.5 min (data not shown). After 30–60 min PacC72 has disappeared yet between 90 and 150 min PacC72 begins to accumulate again, indicating that PacC processing has become limited at the signalling proteolysis step (Fig. 2B). Therefore, the possibility of pH regulation of transcription of the pH regulatory pal genes and the consequences of their overexpression were explored. palA, -B, -C, -H and -I were found to be expressed largely independently of pH and/or the mutational status of other pH regulatory components, i.e., pacC and palF (Denison et al., 1995; 1998; Negrete-Urtasun et al., 1997; 1999). However, palF was found to be an acid expressed gene (Fig. 3A and B). palF transcript levels are highest under acidic conditions and in acidity mimicking mutants and relatively low under alkaline growth conditions and in alkalinity mimicking mutants.

Fig. 3. palF is an acid expressed gene and its overexpression results in alkalinity mimicry. A and B. Northern blots of total RNA probed with P32 labelled palF-specific probe. Methylene blue stained rRNA is a loading control. Strains were grown in MFA-derived media either in steady-state conditions at the pH values shown (A) or shifted from pH 4.3 (H) to pH 8.3 (OH) for up to 6 h (B). Northerns show that palF is preferentially expressed under acidic growth conditions and in the acidity mimicking strains carrying pacC 6309, palB88 and pacC 209 as compared with in the wild type and the alkalinity mimicking pacC14 strains (A) and that palF transcript levels quickly fall on shifting from acidic to alkaline growth conditions (B). C. Progeny from a cross of an argB2 (arginine requiring) strain carrying pacC900 [MYC3-tagged pacC (Peñas et al., 2007)] [pabaA1 yA2 pyrG89 argB2 pacC900 (MP12)] and a strain carrying alcA::palF::argB containing the palF overexpressing cassette driven by the alcohol-inducible alcohol dehydrogenase promoter and integrated at argB to restore arginine prototrophy [alcA::palF::argB pantoB100 (JR110)] are shown. Position 21 argB2 pacC900 (MP12), position 25 alcA::palF::argB (JR110), position 26 biA1 wild-type strain, (C) acid phosphatase stain (Acid phos) on minus phosphate medium containing 1% glucose pH 6.5, (Cii) acid phosphatase stain on minus phosphate medium containing 1% ethanol as carbon source pH 6.5 and (Ciii) synthetic complete glucose medium minus arginine. pacC14 and pacC50, which are strong and moderate pacC constitutive alleles, respectively, are shown below the acid phosphatase plates. Comparison of panels ii and iii demonstrates co-segregation of arginine prototrophy and reduced acid phosphatase levels under alcA inducing (palF overexpression) conditions.
Fig. 3. D. Suppression of palB524 by palF overexpression. Acid phosphatase stained colonies are shown after growth on minus phosphate medium containing 1% glucose (G) or 1% ethanol (E). Repressed levels of acid phosphatase are restored in the palB524 argB+::alcA:palF progeny after growth on ethanol. palB524 is a leaky loss of function allele described by Peñas et al. (2007). The genotype of the palB524 argB+::alcA:palF progeny was checked by sequencing to confirm the palB524 mutation and Southern blot analysis to confirm the presence of the overexpression cassette.

E. Western blot comparing PalF levels and PacC processing in shifted strains containing endogenously or constitutively (gpdA™) expressed palF shows that elevated PalF levels correlate with increased PacC processing and that attenuation of the pH signal, manifest by accumulation of PacC72 with time after shifting, is almost completely removed when palF is overexpressed from the heterologous (gpdA™) promoter. Strains MAD2352 and MAD4500 were used.

F. Petri dish assays show the alkalinity mimicking phenotype of gpdA™:GFP::PalF::pyrO4 palF15 (TM280) (labelled as gpdA™:palF::pyrO4 palF15) as compared with a wild-type (bia1) strain with respect to molybdate resistance and reduced acid phosphatase staining. The pyrO4 palF15 recipient strain (TM261) is also shown. The full genotypes of the strains are described in Experimental procedures.
Moreover, on shifting from acidity to alkalinity, \textit{palF} transcript levels rapidly fall very low between 30 and 120 min after transfer, being somewhat restored after 4 h (Fig. 3B). This transcriptional behaviour resembles the temporal pattern of PacC processing and suggests that expression of \textit{palF} might be rate-limiting in pH signal transduction. This is supported by overexpression studies (Fig. 3C and D) that show that \textit{palF} expression from the strong, inducible \textit{alcA} (alcohol dehydrogenase) promoter results in alkalinity mimicry, as indicated by reduced acid phosphatase expression on ethanol-containing, low phosphate medium, whereas there were no such phenotypic consequences of the overexpression of \textit{palA}, -B, -C, -H or -I (data not shown). In addition, \textit{palF} overexpression is sufficient to suppress the very leaky \textit{palB}24 mutation (Peñas et al., 2007), for acid phosphatase expression (Fig. 3D) and to rescue partially certain leaky mutations in \textit{palH} and \textit{palC} and \textit{palI} (data not shown). \textit{[palI]}32 is a null allele which, like all \textit{palI} mutations, has a less extreme phenotype than non-leaky mutations in the other \textit{pal} signal transduction pathway genes (Denison et al., 1998)). \textit{palF} overexpression from a modified \textit{gpdA} (glyceraldehyde 3-phosphate dehydrogenase) promoter (\textit{gpdA}\textsuperscript{ren}) (Pantazopoulou and Peñalva, 2009) also results in alkalinity mimicry as detected in Petri dish assays (Fig. 3F and Hervás-Aguilar et al., 2010). Western blot analysis demonstrates that \textit{gpdA}\textsuperscript{ren} driven \textit{palF} overexpression results in elevated levels of PacC processing and largely overrides attenuation of pH signalling as manifested by the much reduced levels of PacC\textsuperscript{72} detectable 60 min and 120 min after shifting (Fig. 3E). The appreciable levels of PacC\textsuperscript{72} produced under acidic growth conditions (Fig. 3E) are indicative of pH signalling and suggest that, in agreement with the alkalinity mimicking phenotype, constitutive expression of PalF results in an inappropriate pH response.

These results suggest that the \textit{A. nidulans} pH regulatory response is subject to negative feedback in a fashion similar to that involving \textit{RIM8} of \textit{C. albicans} and \textit{S. cerevisiae} (Porta et al., 1999; Ramon et al., 1999; Lamb and Mitchell, 2003). These results also support some recent mathematical modelling predictions that suggest the presence of a negative feedback loop in the PacC activation process (Ke et al., 2013).

\textbf{pacX: a new gene involved in the pH regulatory response}

\textit{Identification and genetic mapping of pacX}. The first identified \textit{pacX} mutation, designated \textit{pacX}1, was isolated, following UV mutagenesis of a \textit{pacC}\textsuperscript{+}20205 (processing recallitrant) strain, among numerous (largely intragenic) revertants selected for their ability to grow at pH 8.0. \textit{pacX}1, that segregated as a single trait, independently of \textit{pacC}, was localised further to chromosome VIII using parasexual genetics. In view of this localisation and the \textit{pacX}1 phenotype, which includes the partial restoration of alkaline phosphatase biosynthesis, it seemed possible that \textit{pacX}1 is allelic to \textit{suA1palB7}, a mutation linked to the \textit{argC3} translocation breakpoint on chromosome VIII, isolated and characterised by Dorn (1965), during his studies of the phosphatases of \textit{A. nidulans}. Guided by these results, and those of Clutterbuck (1993), \textit{pacX}1 was mapped 8.3 and 16 map units respectively between the \textit{argC3} translocation breakpoint and \textit{hisC38}. Allelism with \textit{suA1palB7} and \textit{suD2palA1}, also isolated by Dorn (http://fgsc.net/Archive/nid.html), was confirmed by further genetic analysis (data not shown) and determination of mutant sequence changes (see below).

\textbf{Characterisation of the pacX mutant phenotype: by-passing pH signal transduction}

\textit{pacX}1 was found to suppress loss-of-function mutations in each of the \textit{pal} pH signal transduction genes, specifically, \textit{palA1}, \textit{palB7}, \textit{palC4}, \textit{palF15}, \textit{palH17} and \textit{palI30}. Phenotype testing of the \textit{pacX}1 \textit{palA}\textsuperscript{+} -B -C -F -H and -I, \textit{pacX}1 \textit{pacC}\textsuperscript{+}20205 (Fig. 4), \textit{pacX}1 \textit{pacC}\textsuperscript{+}207, \textit{pacX}1 \textit{pacC}\textsuperscript{+}209 and \textit{pacX}1 \textit{pacC}\textsuperscript{+}210 (data not shown) double mutants showed that, in addition to partial restoration of growth at pH 8.0 and alkaline phosphatase biosynthesis, \textit{pacX}1 reduces the derepressed levels of acid phosphatase to levels intermediate between those of the \textit{pal}\textsuperscript{+}, \textit{pacC}\textsuperscript{+}20205, \textit{pacC}\textsuperscript{+}207, \textit{pacC}\textsuperscript{+}209 and
pacC−120 strains and those of wild-type strains and pacX1 not only abolishes molybdate sensitivity, it results in moderate molybdate resistance relative to wild-type strains and similar to that observed in pacC− constitutive strains. pacX1 single mutants are alkalinity mimicking, molybdate resistant and have somewhat elevated levels of alkaline phosphatase and reduced levels of acid phosphatase (hence the pac designation) (Fig. 4). In addition, they have slightly reduced conidiation at pH 6.5 and they are resistant to lithium toxicity. Thus, in Petri dish tests, pacX1 single mutants resemble weak pacC− constitutive mutants.

The range of pacC alleles phenotypically modified by pacX− mutations. Numerous pacX mutations have been selected as suppressors of pal− or pacC− processing recalcitrant mutations (Table 1). All extant pacX mutations have the same phenotype and pacX mutant sequence changes clearly show that these are loss-of-function (see, for example, pacX3503 and pacX20, Table 1). The range of pacC alleles affected by pacX− mutations was further explored by phenotype testing of a variety of pacC alleles in combination with pacX− mutations. The results (Table 2) demonstrate that pacX− mutations are unable to suppress pacC− null mutations and severe pacC truncation alleles. However, pacX− mutations enhance the toxicity of the overexpressed alcA::MYC::PacC6-253 allele, which approximates the PacC processed form. In addition, pacX1 is additive with the weak constitutive mutations pacC−234, pacC−39 and pacC−20042; that is, the pacC− pacX1 double mutants are more alkalinity mimicking than the pacC− single mutants (Table 2 and data not shown). The epistasis of pacC− alleles to pacX− alleles strongly suggests that PacC acts downstream of, or is more directly involved than PacX in the pH regulatory response.

Although it appears that a pacX− phenotypic manifestation requires the PacC processed form to be largely functional, not all conforming alleles are affected. For example, the alkalinity mimicry of pacC− 20000 (5-251 + 5) is enhanced, with respect to morphology, by pacX−, but pacC−20601 (5-260) (Mingot et al., 1999), which contains the intact processed form and has a similar phenotype, appears to be unaffected. pacC−69 (L340S) and pacC−50 (5-266) (Tilburn et al., 1995; Mingot et al., 1999; Espeso et al., 2000) do not appear to be phenotypically enhanced by pacX1, possibly because increased alkalinity mimicry in these strong constitutive backgrounds requires sensitivity beyond that of Petri dish tests. Lack of suppression of acidity mimicking mutants pacC−230 (PacC5-238fs) and pacC−206 (PacC5-310fs) (Mingot et al., 1999), which are both suppressed by mutations affecting the proteasomal degradative pathway (JT and HNA, unpublished), suggests that the effects of pacX1 are insufficient to offset this process. Possibly for similar reasons, pacC− 7601 (5-379fs) (Tilburn et al., 1995), which contains a long frameshifted tail, is also unaffected. pacC504 (pacC5) M51 (5-523) (Tilburn et al., 1995), is a muted pacC− allele due to mutation removing the preferred translation start site and lack of additivity (data not shown) suggests that pacX1 is insufficient to compensate for this. Hypostasis (data not shown) of pacX3 to pacC700 pacC− 70001 that prevents PacC27 nuclear import (Fernández-Martínez et al., 2003) implies that suppression by pacX3 requires appropriate localisation of PacC27.

Molecular effects of pacX− mutations. pacX− mutations result in derepression of pacC expression. Northern blots in Fig. 5A demonstrate that, after growth in neutral media, pacX1, pacC−2005 pacX1 and palB7 pacX1 strains all have elevated pacC transcript levels, relative to the respective pacC− strains, similar to those in the pacC−14 strain. In a pH shift experiment (Fig. 5B), the pacX20 strain has constant pacC transcript levels, independent of growth pH and similar to those obtained in the wild-type strain in response to alkalinisation. Thus, pacX− mutants resemble pacC− null (Fig. 1A) and pacC− (Fig. 5A and data not shown) mutations in having derepressed pacC transcript levels.

The effects of pacX1 on pacC transcript levels are reflected in PacC DNA binding activity detected in EMSAs where there are considerably increased amounts of the lower mobility complex containing PacC72 and/or PacC53 from protein extracts of a pacX1 strain relative to those from a wild-type strain from neutral grown mycelia (Fig. S2). The relatively modest increase in the amount of the higher mobility complex, containing PacC27, from the pacX1 strain indicates that pacX1 does not override the palF transcriptional negative feedback loop (Fig. S2A). In pal− or pacC−20205 pacX1 double mutants, which are phenotypically pH independent, there are increased amounts of both the lower and higher mobility complexes relative to those in the pal− or pacC−20205 single mutants (Fig. S2B and C).

Western blot analyses of shift experiments in Fig. 5C, where all three PacC forms can be distinguished, confirm that the increased amounts of complexes detected in EMSAs from the pacX− strains are due to increased amounts of protein, rather than improved binding efficiencies. In addition they demonstrate that prior to the 120 min time point, all PacC forms, where present, are considerably elevated in the pacX20 strain relative to those in the wild type. After 120 min PacC levels in the pacX20 strain have fallen and both the relative proportions and the amounts of the three PacC forms are very similar in both strains.

PacC27 levels are also highly elevated in the palB38 pacX20 strain relative to those in the (null) palB38 single mutant and similar to those obtained in the pacX20 single
mutant under acidic conditions (Fig. 5C). On shifting to alkalinity, levels of PacC72 remain fairly constant in both palB38 strains and appreciable amounts of PacC27 and partial degradation products in the palB38 pacX20 strain are detectable throughout. These bands are attributable to C-terminal, processive, Pal-independent proteolysis of the minor proportion of PacC72 having an ‘open’, and proteasome accessible, conformation that exists in equilibrium with the much more numerous PacC72 molecules that are ‘closed’ and proteasome resistant (Espeso and Arst, 2000; Espeso et al., 2000; Díez et al., 2002; Hervás-Aguilar et al., 2007; Peñas et al., 2007; Peñalva et al., 2008).

Cloning the pacX gene by transposon-mediated mutagenesis.

Extensive attempts to rescue the pacX1 mutant phenotype by co-transformation of an argB2 pacX1 palA1 strain with plasmid pLJ16 carrying argB+ (Johnstone et al., 1985) and chromosome VIII allocated Lorist or pWE15 derived cosmids (Brody et al., 1991) selecting for argB+ and testing for the palA1 alkaline sensitivity phenotype on pH 8.0 medium were unsuccessful. A subsequent PCR analysis suggested that pacX may be absent from these libraries. Transposon-mediated mutagenesis was chosen as an alternative cloning strategy because of the ease with which a pacX− mutation could be selected. This took advantage of available strains carrying the modified impala transposon from Fusarium oxysporum tagged with the yA gene, required for green pigmentation of conidia, inserted in the promoter of the niaD gene thus resulting in the inability to utilise nitrate (Li Destri Nicosia et al., 2001). A yAΔ::Ncypyr4, pabaA1, niaD::impala::yA+, pacC+/−2025 strain was constructed, and transposition was found to occur at a frequency of $\sim 10^{-5}$. Conidia of this strain were spread on pH 7.5 medium with nitrate as sole nitrogen source to select

![Fig. 5. pacX− mutations result in derepressed expression of pacC. (A and B) Northern blots of total RNA are shown. A. The pacC transcript was detected among RNA from a variety of strains grown at neutral pH, pH ~ 6.5, using a 32P-labelled 1041 bp pacC-specific fragment, which had been generated by PCR using the primers BIGFF and TILREV (Table S1). Mycelia were grown for 14 h in shaken minimal medium, containing 1% glucose as sole carbon source and 10 mM 2-(N-morpholino)ethanesulphonic acid, at 30°C. Loading controls were established using a 650 bp Ncol-Ncol fragment from the Aspergillus nidulans actin gene (Fidel et al., 1988). B. Strains were grown overnight (~ 14 h) at 37°C with shaking in acidic MFA and transferred to alkaline MFA for the times shown. Methylene blue stained 18S rRNAs are included as loading controls. pacX+, yA2 pabaA1 pacC900 (J2153) and pacX20, yA2 pabaA1 pacC900 pacX20 (X900A) strains were used. C. Western blots of cell lysates. The 3× MYC tagged pacC+ allele (pacC900) was carried by all strains (Peñas et al., 2007). The strains were pre-grown in acidic media and transferred to alkaline media for the times indicated. pacX+, pyroA4 pacC900 (MAD3877); pacX20, yA2 pabaA1 pacX20 pacC900 (MAD1652); palB38, inoB2 palB38 pacC900 (MAD1362) and palB38 pacX20, pacC900 pantoB100 pacX20 palB38 (MAD4777) strains were used.](image-url)
Out of an estimated 600,000 transposition events, one colony was obtained. The mutant (BG2) was found, by diploid analysis, to contain the insertion on linkage group VIII. This location and the phenotype of BG2 strongly suggested that the insertion had occurred in pacX, as subsequently confirmed (see below). pacX genomic and cDNAs sequences were determined as described in Experimental procedures. 

Table 1. pacX mutations isolated in this work.

| Allele       | Nucleotide change          | Protein change | Mutagen     | Selection       | Reference                          |
|--------------|----------------------------|----------------|-------------|-----------------|------------------------------------|
| pacX12      | +516insimpala              | PPI            | None        | Growth at pH 7.5| This work                          |
| pacX18      | T667insFot1-like transposon | PSD            | None        | Growth at pH 8.0| This work                          |
| pacX24      | +297insC-296-T878          | N275fs, PPI    | None        | Growth at pH 8.0| This work                          |
|             |                            |                |             |                  |                                    |
| Truncating mutations |                       |                |             |                  |                                    |
| pacX3503    | A35insG25-A37             | G12fs          | None        | Growth at pH 8.0| This work                          |
| pacX20      | C133T                      | R44stop        | None        | Growth at pH 8.0| This work                          |
| pacX28      | C180GG                     | P60fs          | None        | Growth at pH 7.5| This work                          |
| pacX8       | ΔC190                      | I63fs          | UV          | Growth at pH 8.0| This work                          |
| pacX13      | ΔG241-243                  | G81fs          | None        | Growth at pH 8.0| This work                          |
| pacX26      | G314-316insG              | R105fs         | None        | Growth at pH 8.0| This work                          |
| pacX34      | G331T                      | H110stop       | None        | Growth at pH 8.0| This work                          |
| pacX19      | (T359-G364)A              | W199fs         | None        | Growth at pH 8.0| This work                          |
| pacX33      | A379-A385insA             | K127fs         | None        | Growth at pH 7.5| This work                          |
| pacX3501    | ΔA445-A449                 | Y148fs         | None        | Growth at pH 8.0| This work                          |
| pacX3502    | ΔC505                      | M168fs         | None        | Growth at pH 8.0| This work                          |
| pacX27      | G657AT                     | D181stop       | None        | Growth at pH 7.5| This work                          |
| pacX31      | C687T                      | R211stop       | None        | Growth at pH 7.5| This work                          |
| pacX4       | (C942-944)insC            | P297fs         | None        | NQO              | Denison and Arst, unpublished      |
| pacX2      | (suA1palB)                 | ΔG945          | G299fs      | None             | Alkaline phosphatase (Dom, 1965)   |
| pacX15      | (G945-951)insA             | G299fs         | None        | Growth at pH 8.0| This work                          |
| pacX351     | (G945-951)insG             | G299fs         | None        | Growth at pH 8.0| This work                          |
| pacX3506    | A951insGGAAGG             | G299GKfs       | None        | Growth at pH 8.0| This work                          |
| pacX11      | ΔC957-G1088               | A301fs         | None        | Growth at pH 8.0| This work                          |
| pacX3512    | ΔAT(108-1090)              | F345fs         | None        | Growth at pH 8.0| This work                          |
| pacX3513    | G1128insC1121-G1128       | Y347fs         | None        | Growth at pH 8.0| This work                          |
| pacX5       | ΔG1250                     | A399fs         | None        | Growth at pH 8.0| Denison and Arst, unpublished      |
| pacX6       | ΔC(1340-1345)              | A430fs         | None        | Growth at pH 8.0| Denison and Arst, unpublished      |
| pacX25      | ΔT1391                     | D246fs         | None        | Growth at pH 8.0| This work                          |
| pacX14      | ΔG1393                     | R447fs         | None        | Growth at pH 8.0| This work                          |
| pacX32      | ΔC(1526-1529)              | P492fs         | None        | Growth at pH 7.5| This work                          |
| pacX3508    | ΔC(1526-1529)              | P492fs         | None        | Growth at pH 8.0| This work                          |
| pacX3509    | G1530insA                 | P492fs         | None        | Growth at pH 8.0| This work                          |
| pacX3507    | ΔC1571, ΔA1572            | S506fs         | None        | Growth at pH 8.0| This work                          |
| Missense mutations |                  |                |             |                  |                                    |
| pacX3      | (suD2palA1)               | C130T          | None        | Alkaline phosphatase | Dom*                           |
| pacX16      | T248C                      | L83P (bs)      | None        | Growth at pH 8.0| This work                          |
| pacX21      | C310A                      | P104T (ef)     | None        | Growth at pH 8.0| This work                          |
| pacX23      | G357T                      | W110G (bs)     | None        | Growth at pH 8.0| This work                          |
| pacX17      | AG458-G460                 | R153S (ef)     | None        | Growth at pH 8.0| This work                          |
| pacX3505    | G679C                      | R209P (ef)     | None        | Growth at pH 8.0| This work                          |
| pacX22      | G700C                      | R216P (ef)     | None        | Growth at pH 8.0| This work                          |
| pacX9       | G715C                      | R221P (ef)     | None        | NQO              | Denison and Arst, unpublished      |
| pacX11      | C721G                      | A225G (ef)     | None        | None             | This work                          |
| pacX7       | C198AT                     | P444L (ef)     | None        | Molybdate resistance | This work                        |
| pacX30      | G1396T                     | C448Fs (bs)    | None        | Growth at pH 7.5| This work                          |
| pacX29      | G1468T                     | C472F (bs)     | None        | Growth at pH 7.5| This work                          |
| pacX10      | P1470C                     | S473P (ef)     | None        | UV               | Akintade and Tilburn, unpublished |

Mutation isolated as suppressor of: 1pacC−20205, (Díez et al., 2002), 2pacC−207 (Vincent et al., 2003), 3pacF−20 (Arst et al., 1994), 4pacI30 (Denison et al., 1998), 5palB7 (Dom, 1965; Peñas et al., 2001), 6palA1 (Dom, 1965), 7palA9 (Arst et al., 1994; Denison et al., 1998), 8pacC209 (Díez et al., 2002), 9pacC900insimpala (Peñas et al., 2007). Mutations were isolated in pacX5 which is an S-tagged pacX allele (PacX::4GA::S-tag, see Experimental procedures). PPI, predicted promoter insertion; PSD, predicted splicing defect. The ConSurf (http://consurf.tau.ac.il/) predictions for substituted residues are given in parentheses: ef, exposed and functional; bs, buried and structural. See Experimental procedures for media.

Simultaneously for pacX mutations and restoration of the niaD genotype by excision of the impala transposon.

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Table 2. Modified and unmodified pacC allele phenotypes.

| pacC allele | Phenotype | PacC mutant protein (unprocessed form) | Effect of pacX in double mutant |
|-------------|-----------|---------------------------------------|--------------------------------|
| Modified phenotype | | | |
| pacC<sup>207</sup><sup>a</sup> | Acidity mimicry | Y455N | Suppression |
| pacC<sup>209</sup><sup>b</sup> | Acidity mimicry | L498S | Suppression |
| pacC<sup>210</sup><sup>b</sup> | Acidity mimicry | L498F | Suppression |
| pacC<sup>20205</sup><sup>5</sup><sup>a</sup> | Acidity mimicry | 5-464 IDRPGSPL 541-678 | Suppression |
| pacC<sup>39</sup><sup>5</sup> | Weak alkalinity mimicry | L266F | Additivity |
| pacC<sup>234</sup><sup>a</sup> | Weak alkalinity mimicry | L340F | Additivity |
| pacC<sup>20042</sup><sup>b</sup> | Weak alkalinity mimicry | R579T | Additivity |
| pacC<sup>20002</sup><sup>b</sup> | Acidity mimicry | Q155K | Enhanced loss-of-function phenotype |
| pacC<sup>20000</sup><sup>5</sup> | Neutrality mimicry | 5-251 + 5 | Additivity |
| alaC<sup>5</sup>:MYC::PacC<sup>6-253</sup><sup>a</sup> | Alkaline mimicry | 5-252 | Increased toxicity |
| No modification of phenotype | | | |
| pacC<sup>Δ::Ncpy4</sup> | Null | | |
| pacC<sup>6310</sup><sup>a</sup> | Null | 5-163 | |
| pacC<sup>7804</sup><sup>a</sup> | Acidity mimicry | 5-173 + 9 | |
| pacC<sup>230</sup><sup>5</sup> | Acidity mimicry | 5-238 | |
| pacC<sup>700</sup> pacC<sup>Δ::70001</sup><sup>1</sup> | Acidity mimicry | GFP::5-250 K159M | |
| pacC<sup>20601</sup><sup>1</sup> | Neutrality mimicry | 5-260 | |
| pacC<sup>50</sup> | Alkaline mimicry | 5-266 | |
| pacC<sup>206</sup> | Acidity mimicry | 5-310 | |
| pacC<sup>69</sup> | Alkaline mimicry | L340S | |
| pacC<sup>7601</sup> | Acidity mimicry | 5-379 | |
| pacC<sup>504</sup>, pacC<sup>58</sup> | Weak neutrality mimicking | 1-523 | |

The pacX allele is indicated after the pacC allele number: *pacX<sup>20</sup>, <sup>b</sup>pacX<sup>1</sup>, *pacX<sup>3</sup>. The pacC<sup>20205</sup> allele also includes pacC<sup>202</sup>. Allele alaC<sup>5</sup>:MYC::PacC<sup>6-253</sup> specifies M-MYC(EQKLISEEDL)-AAAS-PacC<sup>6-253</sup> and is integrated in pyrA in an otherwise pacC null background.

PacX sequence

The pacX sequence specifies a 661 residue protein. A zinc binuclear cluster DNA binding motif is located towards the carboxy-terminus between residues 445 and 472 and a region predicted to form a coiled-coil structure occurs in the region of residues 178 to 224 (Fig. 6 and Fig. S3). In BLAST searches of the databases, the region containing the putative DNA binding domain and the amino-terminus extending to residue 228 are the most highly conserved (see below), indicating their functional importance (Fig. 6, Figs S3 and S4). Different algorithms all predict a nuclear localisation for PacX; ngLOC (17.6% nuclear 14.5% cytoplasmic), PSORT II (73.9% nuclear) with cNLS mapper predicting highly probable monopartite (TPGKRPRESSGEF, residues 10 to 22, score 6.5) or bipartite (ETPGKRPRRESSGEFPPASKVPKT, 9 to 32, score 9.0) NLSs. An NES (nuclear export sequence) is predicted with high probability by NetNES between residues 179 and 191 (Fig. 6).

The coiled-coil prediction for A. nidulans PacX is 1 and therefore very strong (Fig. S3). The distancing of the coiled-coil region from the zinc binuclear cluster is somewhat conserved among PacX homologues (Fig. 6 and Fig. S3). Coiled-coils are putative oligomerisation domains, and the role of the coiled-coil is supported by the partial dominance of pacX<sup>1</sup> vis a vis pacX<sup>3</sup> (Fig. S5). pacX<sup>1</sup> results in a frameshift after residue 301 (Table 2), thus conserving the coiled-coil element but deleting the Zn cluster. A number of point mutations map within the putative coiled-coil element (Figs S3 and S4 and Table 1).

The PacX putative DNA binding domain (Fig. 6) conforms to the consensus sequence of zinc binuclear cluster motifs (Todd and Andrianopoulos, 1997; MacPherson et al., 2006) characteristic of, even if not completely exclusive to, fungi (Scacciozzi, 2014). Zinc binuclear cluster DNA binding motifs are almost universally at the amino-terminus of cognate transcription factors (MacPherson et al., 2006); however, that of PacX is towards the carboxy-terminus, which resembles S. cerevisiae Ume6p (Strich et al., 1994) and C. albicans Czf1p (Whiteway et al., 1992 reviewed by MacPherson et al., 2006). Infrequent but not unique to PacX and its orthologues are the two Pro residues N terminal to the first Cys, and the absence of a Pro residue one or two residues N-terminal to the fourth cysteine (Fig. S4). The dimerisation element is usually in Zn-cluster proteins a few residues C-terminal to the DNA binding motif, and it is typically shorter than the one seen in PacX, such as 15 residues for Gal4, 19 residues for Ppr1 (Marmorstein et al., 1992; Marmorstein and Harrison, 1994) with the maximal length predicted for such a C-terminal coiled-coil dimerisation domain being 25 residues (Schjerling and Holmberg, 1996). This contrasts with the 46 residue-long putative coiled-coil, 221
residues amino-terminal to the first Cys of the Zn cluster extant in PacX. It could be proposed that while the function of the standard dimerisation domain found in Zn binuclear cluster proteins is to permit the recognition of DNA inverted, everted or direct repeats separated by a few base pairs [e.g. 11 in Gal4, 6 in Ppr1 and UaY (Marmorstein et al., 1992; Marmorstein and Harrison, 1994; Suarez et al., 1995)], the probable dimerisation of PacX may serve an altogether different function.

**Phylogenetic analysis**

Searching with blastp using the PacX protein sequence as *in silico* probe of the 431 fungal genomes available in the Joint Genetics Institute database on the 30th of January 2015 showed PacX homologues to be present in the Pezizomycotina and not in any other fungal taxon, but being absent from the two sequenced genomes of the Orbiliomycetes and the seven sequenced genomes of the Pezizomycetes, basal classes of the Pezizomycotina. Absence from these and other ascomycete taxons (Saccharomycotina and Taphrynnopmycotina) was confirmed with a tblastn search. Searching the NCBI protein database, excluding the Pezizomycotina, failed to reveal any homologues with either blastp or tblastn. Although within the Eurotiomycetes the sequence is very conserved, the phylogenetic tree (Fig. S6) and the alignment (Fig. 6 and Fig. S4) demonstrate that there is much divergence within the Pezizomycotina. Within the Sordariales, the sequence even within the zinc binuclear cluster is divergent, as shown by the *Neurospora crassa* sequence in the alignment (Fig. 6). Within the Dothidiomycetes, the sequences diverge even more and form two clusters roughly corresponding to the Pleosporales and Capnodiales with one
outgroup (see legend to Fig. S6 for details), secondary loss has occurred in several species, mainly within the Sordariales, including Magnaporthe grisea and Podospora anserina (confirmed by tblastn).

Mutant sequence changes

Forty-five mutant sequence changes confirm the identity of the gene (Table 1). Clustering of missense mutations exclusively to within the amino-terminus and the predicted coiled coil region itself and the zinc binuclear cluster or immediately adjacent to it underscores the functional importance of these regions (Fig. S4). The results of comparing PacX with the consensus derived from an alignment of 177 PacX homologues using the ConSurf algorithms (http://consurf.tau.ac.il/) for the identification of functional regions in a protein are presented in Fig. S4, and the predictions for mutated residues are summarised in Table 1. All of the missense mutations affect conserved residues that occur in conserved regions (Fig. S4). Three out of four mutations in the coiled-coil pacX3505(R209P), pacX22(R216P) and pacX9(R221P) are basic to non-polar. These are changes of Arg to Pro, a residue that breaks α-helices. However, the coils/pcoils algorithm (http://toolkit.tuebingen.mpg.de/pcoils) only revealed in each case minor changes for the probability or length of the predicted coiled-coil. All mutations within this region [which includes pacX11(A223G)] change conserved residues that are predicted to be exposed and functional and may affect a specific interaction of PacX. The majority of truncating mutations remove conserved regions of the protein. Even more extreme truncations, pacX3503 and pacX20, terminate the protein at residues 12 and 44, respectively, thus removing almost all of the protein, confirming that these classically obtained pacX alleles are complete loss-of-function (Table 1). The absence of mutations and poor conservation in the C-terminus suggested that this region might be dispensable. This was confirmed with an engineered allele expressing PacX residues 1 to 499 from the alcA promoter (alcA::PacX1-499) and integrated at pyroA, which was found to be as functional as the full-length protein expressed from the same promoter (alcA::PacX1-661) at the same integration site. pacX3507, which terminates the protein after Ser506 and therefore retains the functional region, has a long (41 residue) out-of-frame tail that might destabilise the protein or interfere with its activity and thus cause the loss-of-function phenotype.

pacX mutations also include the intergenic duplication in pacX24 and the impala transposition into the pacX promoter of pacX12. In addition, pacX18 was found to be an insertion identical to the F0t1-like transposon in chromosome VIII defined by AN0826 (encoding the transposase). Surprisingly, sizing of the region of AN0826 amplified using external primers shows the transposon to be retained at its resident primers shows the transposon to be retained at its resident locus in the mutant strain (see Supplementary Data).

PacX nuclear localisation

The PacX sequence, which contains putative nuclear localisation signals and a putative zinc binuclear cluster DNA binding domain, strongly suggested that PacX functions in the nucleus. PacX localisation was investigated by live epifluorescence microscopy of a strain expressing PacX::GFP and HhoA::mCherry (histone 1). Fig. 7 shows that PacX::GFP is localised within the nuclei where it forms one strongly fluorescent, discrete spot per nucleus. Shifting from acidity to alkalinity for 1 h had no effect on PacX::GFP localisation that therefore appears to be pH independent (Fig. 7). PacX localisation resembles that of the proline degradation pathway-specific zinc binuclear cluster transcription factor PrmA, which has proline-independent, sub-nuclear localisation (Pokorska et al., 2000).

Discussion

PacX is a protein of unusual domain structure, with a conspicuous coiled-coil helix amino-terminal to a typical carboxy-terminal fungal Zn cluster. The pH signalling pathway, including the central transcription factor PacC/Rim101, is conserved throughout the ascomycetes and, to a certain extent, in the basidiomycetes and has been studied in ascomycetous yeasts and basidiomycetes (Peñalva et al., 2008; Davis, 2009; Selvig and Alspaugh, 2011; Blanchin-Roland, 2013; Comet and Gaillardin, 2014; Obara and Kihara, 2014; Peñalva et al., 2014; Herrador et al., 2015; Ost et al., 2015, and references therein) (in addition to A. nidulans and other filamentous ascomycetes). However, the additional putative transcription factor PacX is an evolutionary novelty. It occurs only in the Pezizomycotina and within the limits of the paucity of sequences available, particularly in the Orbiliomycetes, it seems to have appeared after the divergence of the other classes of the Pezizomycotina (Leotiomyceta) from the Pezizomycetes and the Orbiliomycetes. Recent studies, including those combining fossil and molecular, data differ as to whether the Pezizomycetes or the Orbiliomycetes constitute the most basal group of the Pezizomycotina (Schoch et al., 2009; Prieto and Wedin, 2013; Beimforde et al., 2014). However, these studies agree that the Leotiomyceta have diverged from the other two classes most probably in the Silurian era (∼430 Mya). Thus, the appearance of PacX correlates with a major phylogenetic split, which may coincide with the establishment of biotic interactions with vascular plants (Prieto and Wedin, 2013).
The characterisation of mutations in \textit{pacX} has pinpointed the crucial functional regions, which correlate with those conserved throughout the Leotiomyceta. One mutation however is quite unique: In more than 60 years of \textit{A. nidulans} genetics, this is the first and only reported mutation resulting from an insertion of an endogenous transposon. What is even more surprising is that the \textit{Fot1} like element defined by AN0826 did not transpose by a mechanism of cut and paste, characteristic of the transposons of this class, including the heterologous transposition of the \textit{F. oxysporum} \textit{Fot1} transposon in \textit{A. nidulans} (Li Destri Nicosia et al., 2001 and refs therein) but rather by a copy and paste mechanism (see Supplementary Data).

Proposed role(s) of PacX together with negative autoregulation of \textit{pacC} and the negative feedback acting on \textit{palF} are incorporated in a model for the control of the alkaline pH response, which is shown in Fig. 8. PacC\textsuperscript{72} exists predominantly in a closed proteasome inaccessible conformation in equilibrium with a small fraction of molecules assuming a less favourable open, proteasome-accessible conformation, thus providing a substrate for the Pal-independent bypass. In the absence of pH signal transduction, PacC\textsuperscript{72} is largely unprocessed (Figs 2 and 5C) and, with the participation of PacX, represses its own transcription, as demonstrated by the increased \textit{pacC} transcript levels obtained in \textit{pacC} null or \textit{pacX} null strains (Figs 1A and 5B). This would reduce substrate-driven flux through the Pal-independent pathway. However, overexpression of \textit{pacC} by itself produces no detectable phenotype, and overexpressing \textit{pacC} alleles expressed from the \textit{alcA} or \textit{gpdA\textsuperscript{mini}} promoter are hypostatic to \textit{pal} mutations (Fig. 9 and Mingot et al., 2001). In contrast, \textit{pacX} mutations result in alkalinity mimicry, and they are able to suppress \textit{pal} and processing recalcitrant \textit{pacC}\textsuperscript{72} mutations. Moreover, they are able to suppress \textit{pal} mutations even when \textit{pacC} is expressed from the heterologous \textit{alcA} (Fig. 9) and \textit{gpdA}\textsuperscript{mini} (data not shown) promoters, i.e.

\textbf{Fig. 7.} Sub-nuclear localisation of PacX.
A. Image of a hyphal tip cell coexpressing PacX-GFP and HhoA::mCherry (histone 1) to label chromatin. DIC, Nomarski optics.
B. Top, individual planes of a z-stack of images, acquired with the indicated Z-distance. Bottom, maximal intensity projection (MIP) of the above stack and orthogonal [(X, Z) and (Y, Z)] views across the indicated lines. All images were captured from cells cultured in acidic medium. Exposure to alkaline media for 60 min did not alter this PacX sub-nuclear localisation pattern.
inhibition of PacC27 activities also potentially explains why the very small amounts of PacC27 present in pacX pal− strains (Fig. 5C) are able to suppress the pal− phenotype to such an appreciable extent (Fig. 4). The alkalinity mimicking phenotype of pacX mutations that result in elevated alkaline phosphatase (palD) and reduced acid phosphatase (palA) levels demonstrates that PacX affects both activator and repressor functions of PacC27 and that it is required for normal responses to acidity and neutrality.

The onset of PacC processing occurs less than 4 min after exposure to alkalinity (Fig. 2A), and this rapid response would be facilitated by the pool of PalF accumulated under acidic conditions, due to palF being acid expressed (Fig. 3A and B). After approximately 1.5–2 h, steady-state conditions are established and PacC72 begins to accumulate again (Fig. 2B), indicating that the signalling proteolysis has become limiting and that the signal has become attenuated. Work presented here, wherein overexpression of palF from a heterologous promoter largely prevents accumulation of PacC27 in alkaline media strongly suggests that repression of palF transcription, directly or indirectly, by PacC53 or PacC27 plays a major role in this attenuation. It appears that there is a relatively modest fall in PalF protein levels as compared with the fall in palF transcript levels, in response to alkalisation. This suggests that attenuation is not a function of absolute PalF amounts but rather that of the rate of de novo PalF synthesis. Low rates of PalF synthesis might inhibit Pal signalling, for example, by limiting the rate of the crucial PalF ubiquitylation. Other modifications might be affected. In addition to ubiquitylation, A. nidulans PalF is phosphorylated in an alkaline pH dependent fashion (Herranz et al., 2005). Interestingly, CK1-mediated phosphorylation of S. cerevisiae Rim8 has recently been reported to prevent Rim signalling (Herrador et al., 2015). Thus, attenuation might be achieved by limiting the rate of de novo, unphosphorylated, pathway-activating PalF. An even more tempting scenario would be that the degree of signalling is a balance between the levels of ubiquitylated and phosphorylated PalF. In this situation, the effects of small changes in de novo synthesised PalF would be additive and could well account for the quite profound effects observed on overexpression of PalF (Fig. 3E and F).

Thus, control of the alkaline pH response ensures that both the response to alkalisation is Pal signal transduction dependent and an escalating alkaline pH response is prevented by attenuation of pH signalling by negative feedback via repression of palF transcription by PacC53 and/or PacC27. It is tempting to speculate that the absence of PacX in yeasts correlates with the absence of pH signal independent activation of the pH responsive transcription factor. In S. cerevisiae Rim101 processing occurs in one step and is entirely Rim signal transduction dependent (Li and Mitchell, 1997; Lamb et al., 2001; Xu and Mitchell,
et al (Lamb et al., 2009; Wang and Szaniszlo, 2009), Trichoderma harzianum (Trushina et al., 2013), but not, curiously, S. cerevisiae (Landraud et al., 2013), Metarhizium robertsii (Lambert et al., 2015), which are also preferentially expressed in acidic media, has been reported (Porta et al., 1999; Ramon et al., 1999; Lamb and Mitchell, 2003), suggesting that negative feedback limiting the arrestin component of the pathway might be a general feature of the pH response. However, in contrast to PalF, where ubiquitylation is strictly pH- and PalH-dependent and pivotal in the response, S. cerevisiae Rim8 ubiquitylation appears to be pH independent and takes place in a double rim21Δ dfg16Δ mutant background, lacking both PalH homologues. However, the role of Rim8 ubiquitylation in Rim signalling becomes clear if the Vps23-Rim8 interaction is debilitated by mutation of a SPX motif in the latter (Herrador et al., 2010). C. albicans Rim8 undergoes hyperphosphorylation, instead of ubiquitylation, in a manner linked to Rim101 processing (Gomez-Raja and Davis, 2012).

Many pacC/Rim101 homologues, like A. nidulans pacC ([Tilburn et al., 1995] and Figs 1 and 5B), are preferentially expressed under neutral to alkaline conditions. These include those of: Beauveria bassiana (Zhou et al., 2014), C. albicans (Bensen et al., 2004), F. oxysporum (Caracuel et al., 2003), Magnaporthe oryzae (Landraud et al., 2013), Metarhizium robertsii (Huang et al., 2015), Trichoderma harzianum (Moreno-Mateos et al., 2007), T. virens (Trushina et al., 2013), Wangiella (Exophiala) dermatitidis (Wang and Szanszlo, 2009), Y. lipolytica (Lambert et al., 1997) but not, curiously, S. cerevisiae (Lamb et al., 2001; Serrano et al., 2002; Lamb and Mitchell, 2003; Viladevall et al., 2004). This suggests that autoregulation might be a common feature, but the absence of reporter studies precludes determining whether this would be positive or negative.

The mechanism(s) of PacX action is/are not clear but what appears to be dual functionality could be achieved by a single mechanism. For instance, if PacC27 and PacC27 were to compete for nuclear import and/or DNA binding, PacX antagonism of PacC27 could fulfil both functions. This might involve protein–protein interactions between PacX and PacC, possibly via the PacX coiled-coil domain and/or direct DNA binding of PacX through the zinc binuclear cluster. PacX localisation, which is almost exclusively nuclear, supports a mechanism occurring within the nucleus.

Investigations into pacC and palF promoter occupancy, the possibility of PacC and PacX interaction, PacX sub-

nuclear localisation and post-transcriptional regulation of PalF are topics for future research.

Experimental procedures

A. nidulans strains, phenotype analysis, genetic techniques and growth media

Aspergillus nidulans strains carried previously described markers, in general use; standard media, phenotype testing and genetic techniques were used (Caddick et al., 1986; Clutterbuck, 1993; Arst et al., 1994; Tilburn et al., 1995 and references therein). LiCl and MoO4 plates were prepared by the addition of lithium chloride or sodium molybdate solution to appropriately supplemented minimal medium (Cove, 1966) containing 1% D-glucose and 5 mM ammonium tartrate, to the desired concentration (100–500 mM LiCl and 25 mM MoO4). Neomycin containing plates were prepared by the addition of neomycin sulphate powder to 1 or 0.5 mg ml–1 to molten minimal medium minus glucose, containing 1% D-glucose or 1% ethanol (added after autoclaving), respectively, and 5 mM ammonium tartrate, pH 8.0 medium followed (Cove, 1976). Dropout media were prepared from fully supplemented yeast Dropout medium (Clontech) containing additional supplements appropriate for the auxotrophies of the strains, 5 or 10 mM urea as nitrogen source and 1% D-glucose, added after autoclaving. Acidic dropout medium and MFA (Peñas et al., 2007), which contained 5 mM ammonium tartrate as nitrogen source, were buffered with 50 mM citrate to give pH 4.3, and alkaline dropout medium and MFA were buffered with 100 mM HEPES to give pH 8.3. MFA was also buffered to give pH 5.6 with 100 mM NaH2PO4 (plus 50 mM NaCl); pH 6.8 with 50 mM NaH2PO4 and 50 mM Na2HPO4 (plus 50 mM NaCl) and pH 7.9 with 100 mM Na2HPO4. To obtain conidiospores for transposon mutagenesis strain 2431A (see below Transposon mutagenesis) was grown on minimal media (see above) with 10 mM NaNO2 as sole nitrogen source, buffered to pH 6.5 with 50 mM MES. To select simultaneously for growth on nitrate as sole nitrogen source and for loss-of-function in pacX, we used pH 7.5 medium, minimal medium (see above Cove, 1966) buffered to pH 7.5 by addition of 50 mM phosphate buffer (made from stock solutions 500 mM Na2HPO4 and 500 mM NaH2PO4), containing 10 mM NaNO2 as sole nitrogen source and 10 × PABA (40 µg ml–1 4-aminobenzoic acid).

Construction of strains

Plasmid pALC-argB (BglII) (Mingot et al., 1999) was used for the construction of pacC and pal overexpression cassettes containing cDNA of pacC and one of each of the pal signal transduction genes (Denison et al., 1995; 1998; Tilburn et al., 1995; Maccheroni et al., 1997; Negrete-Urtasun et al., 1997; 1999) under alcA (alcohol dehydrogenase promoter) control. This plasmid contains a functional alcA, containing a transcription start site, separated from the trpC terminator by a polylinker, and a mutant argB gene to direct integration to argB by repair of the argB2 allele. pal overexpressing strains were constructed by DNA-mediated transformation (Tilburn et al., 1995) of double mutants, argB2 and appropriately pal for the corresponding overexpressed gene, with selection for argB2.

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transfectants, screening for the pal phenotype and Southern blot analysis to identify appropriate single copy integrants. Appropriate pal transfectants were subsequently crossed to obtain pal overexpressing strains in otherwise pal genetic backgrounds. alcA-driven PacC5-678 overexpressing strains were obtained by transformation of strain MAD397 yA2 argB2 palA1 pacCa::Ncpyr4 pantoB100 with [alcA::PacC5-678], as described by Mingot et al. (1999), to give transformant yA2 palA1 argB::alcA::PacC5-678 pacCa::Ncpyr4 pantoB100 (MAD4015), which was crossed to HB85 pabaA (yA2 or yAΔ::Ncpyr4) argB2 pacX20 to give strains (9-1) (yA2 or yAΔ::Ncpyr4) (pyrG897) argB::alcA::PacC5-678 pabaA1 pacCa::Ncpyr4 (9-21) pabaA1 (yA2 or yAΔ::Ncpyr4) (pyrG897) alcA::alcA::PacC5-678 pacCa::Ncpyr4 pantoB100 (Fig. 9).

The pacX gene was 3’ tagged with GFP and S-tag using spacer-GFP/S-tag-AfpGrG cassettes (Yang et al., 2004) and introduced by gene replacement into a nkuA::bar recipient strain (KUG4). KUG4 pyrG897 pyro4A niiA4 nkuA::bar was constructed by crossing to a strain nkuA::bar niiA4 bia1 pyr44 (kindly provided by Prof Michael Hynes). The S-tagged pacX allele was denoted pacX35. Strain AL02 pacX::GFP::AfpGrG (pyrG897) pyro4A Hh0A::mCherry niiA4 nkuA::bar was obtained by crossing transformant A1 pyrG897 pyro4A niiA4 nkuA::bar::pacX::GFP::AfpGrG with strain LO1421 containing Hh0A::mCherry (kindly provided by Prof Beri Oakley).

Strains pabaA1 yA2 gpdA**:GFP-PacC(5-678)::pyroA pacCa::Ncpyr4 (MAD1713) and pabaA1 yA2 gpdA**::GFP-PacC(5-251)::pyroA pacCa::Ncpyr4 (MAD1710) were obtained by transformation of a pabaA1 yA2 pacCa::Ncpyr4 pyro4A recipient strain with plasmids pMD176 and pMD166, respectively, and identification of transformed clones carrying single-copy integration events at pyroA by Southern blotting. pMD176 and pMD166 encode GFP-PacC(5-678) and GFP-PacC(5-251) fusion proteins respectively. Coding regions were obtained as HindIII-EcoRI fragments by PCR, using templates described in Mingot et al. (1999), and introduced into the multiple cloning site of pGpd003 (Pantazopoulos and Peñalva, 2009), downstream of the gpdA** promoter. Strains J2422 yA2 gpdA**:GFP-PacC(5-678)::pyroA pacCa::Ncpyr4 pacC630 pacC609 (pacC63) pantoB100 and J2427 gpdA**::GFP-PacC(5-251)::pyroA pacCa::Ncpyr4 pacC630 (pacC63) were derived by crossing MAD1713 and 1710 respectively to J2384 areA5 pyro4A pacC630 (pacC63) pantoB100 and the pacC630 allele was detected among the progeny by PCR. TM280 pabaA1 gpdA**::GFP::PalF::pyroA palF15 was obtained by transformation of a donor recipient strain TM261 pabaA1 pyro4A palF15 with pTM9015. pTM9015 was derived from p1673 by QuikChange mutagenesis (Agilent Technologies) using primers p1673-EcoRI-Fw and p1673-EcoRI-Rv (Table S1) to introduce an EcoRI site into which PalF CDNA was inserted in place of pacC.

The construction of endogenously expressed MYC3-tagged PacC (allele name pacC900) is described by Peñas et al. (2007) and endogenously HA3-tagged PalF (allele name palF500) or HA3-tagged PalF expressed from gpdA** promoter are described by Hervás-Aguilar et al. (2010). Strain MAD2352, wA4 pyro4A inoB2 palF::HA3::pyrGum pyrG89 nkuA::bar pacC900 was obtained by transformation of MAD1732 (Hervás-Aguilar et al., 2010). MAD4500, yA2 pabaA1 pantoB100 pyroA4::[pyroA*::gpdA**::palF::HA3] pacC900 nkuA was derived by crossing MAD3319 yA2 pabaA1 pantoB100 and MAD3007 pantoB100::[pantoB*::gpdA**::palF::HA3] pyroA4::[pyroA*::gpdA**::palF::HA3] pacC900 ΔnkuA::bar?.

**Northern blots**

Growth conditions for Northern blot are given in the appropriate Fig, legends. RNA was extracted as described by Tilburn et al. (1995) or by a modified Drosophila procedure http://www.koko.gov.my/CocoaBioTech/RNA%20isolation23.html#procedure, as follows. Lyophilised mycelium was ground in a 2 ml tube with a glass rod and mixed with 800 μl of GHCl solution (5 mM DTT, 7.5 M guanidium hydrochloride (Sigma), 25 mM sodium acetate, pH 7.0, 0.5% N-lauryl sarcosinate) and shaken vigorously with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) or acidic (pH 4.5) phenol:chloroform:isoamylalcohol (125:24:1). After centrifugation, the RNA was precipitated from 400 μl of the aqueous phase with 16 μl of 1M acetic acid and 400 μl of ethanol with incubation at −80°C for 1–2 h. The RNA was pelleted by centrifugation and resuspended in 400 μl of GHCl solution and precipitated as before. The RNA was washed with 100% ethanol, then 75% ethanol, air dried and resuspended in RNase free water and stored at −80°C. Northern blotting followed Tilburn et al. (1995). Heat or UV fixed membranes were stained with methylene blue (Sambrook et al., 1989) and probed with appropriate radiolabelled (32P) or digoxigenin (DIG) labelled (Roche) DNA fragments. pacC-specific probes were prepared from PCR fragments obtained with primers (Table S1) TILREV and 1217FF or BIGFF (32P labeled) or 850U and 1217FF (DIG labeled, Fig. 1B). Loading controls were established using a 32P labelled ~650 bp Ncol-Ncol fragment from the A. nidulans actin gene (Fidel et al., 1988) or methylene blue stained 18S rRNA, as indicated.

**Western blots**

Mycelia were cultured and sampled essentially as described by Galindo et al. (2012) except that 5 mM ammonium tartrate was used as nitrogen source and alkaline medium was buffered to pH 8.0 with 100 mM HEPES. Lyophilised mycelia were homogenised, using a 5 mm ceramic bead, following Hervás-Aguilar et al. (2010). Cell lysis followed a method adapted from an S. cerevisiae protocol (Stimpson et al., 2006). 6 mg samples of powdered biomass were weighed and transferred to 2 ml microcentrifuge tubes. Proteins were solubilised in 1 ml tube−1 lysis solution (0.2 M NaOH, 0.2% (v/v) β-mercaptoethanol with vigorous vortexing. Proteins were precipitated with 7.5% trichloroacetic acid (TCA) and pelleted by centrifugation at 14 000 × g for 5 min at 4°C. Pellets were solubilised in 100 μl Tris base, mixed with 200 μl of Laemmli buffer (Laemmli, 1970) and incubated at 100°C for 2 min. Proteins (5–10 μl of each sample were resolved in 8% SDS-polyacrylamide gel, transferred to nitrocellulose membranes. For MYC::PacC and actin blots were reacted with either mouse monoclonal anti-c-myc (at 1/2,000 dilution) (Clone 9E10, Sigma-Aldrich) or mouse anti-actin monoclonal
antibody (1/4,000) (Clone 4, MP Biomedicals, LLC). Peroxidase-conjugated goat anti-mouse IgG immunoglobulin (Jackson) at 1/5,000 and 1/8,000 respectively were used. PalF::HaA blots were reacted and developed as described by Hervás-Aguilar et al. (2010). Peroxidase activity was detected with Amersham Biosciences ECL.

Transposon mutagenesis

pacX was cloned by tagging it with the impala transposon form F. oxysporum in a manner similar to that employed to tag the azaA (Cecchetto et al., 2004) and rrmA (Olszewska et al., 2007). Starting from strain CS2778 (Li Destri Nicosia et al., 2001), which contains an impala yaA tagged element interrupting the niaD promoter, we obtained strain 2431A, yaA::Ncpyr4, pabaA1, niaD::impala::yaA pacC::−20205 by crossing. The impala excision frequency in strain 2431A was determined to be of the order of 10−6, similar to that reported for strain CS2778 (Li Destri Nicosia et al., 2001). Strain 2431A is unable to grow on nitrate by virtue of the impala element and its insertion in pacX, which would suppress the phenotype of pacC−/−20205. Conidiospores of strain 2431A were obtained on minimal media with NaNO3 as sole nitrogen source and plated by top layering on the appropriate selective media (NaNO3 as sole nitrogen source, pH 7.5, see above). One putative strain (BG2) carrying a transposon insertion in pacX was selected as able to grow in this medium. Based on the excision frequency of strain 2431A and the number of viable conidiospores plated, we calculated that ~ 600 000 niaD colonies were generated in the experiment that yielded the putative pacX insertion.

Determining DNA sequence flanking the impala insertion site

Genomic DNA of strain BG2 was isolated and used as template to obtain DNA sequence flanking the impala insertion site by PCR using a single primer YA1 and cycling conditions as described by Karyshev et al. (2000). The major fragment of ~ 0.9 kb was gel purified and sequenced using oligo YA2. The fragment was re-amplified using primer YA1, digested with BamHI (to obtain fragments having a single YA1 sequence), and the gel-purified major fragment of ~ 650 bp was sequenced using primer YA1. These sequencing reactions combined gave 708 bp of sequence flanking the insertion site. Primer sequences are given in Table S2.

Determining the pacX genomic and cDNA sequences and pacX mutant sequence changes

BLAST search of the (then unfinished) A. fumigatus genome database (Nierman et al., 2005) suggested a possible A. fumigatus homologous gene having a C-terminal zinc binuclear cluster. A larger A. nidulans pacX fragment was PCR amplified using the pacX-specific primer XF2 and a degenerate primer ZNF2 based on the sequence of the zinc binuclear cluster of the putative A. fumigatus homologue. In the first three cycles, an annealing temperature of 45°C was employed, followed by 30 cycles at 55°C; 20 pmoles of XF2 and 200 pmoles of ZNF2 were used per reaction. The resulting ~ 1.6 kbp fragment was gel purified and sequenced. A DNA fragment made by PCR using primers XF5 and XR5 was used to isolate pacX clones from a λ gt10 cDNA library (Osmani et al., 1988). Two cDNA clones contained the entire pacX coding sequence and sequencing confirmed the presence of one intron. Nucleotide sequence of the 5’ region of the pacX gene (upstream of the impala insertion and cDNA sequence) was obtained following an ‘inverse’ PCR strategy. Essentially, DNA of BAC clone 28C10 obtained from an A. nidulans BAC library prepared by Ralph Dean and obtained from https://www.genome.clemson.edu/online_orders?&page=productGroup&service=bacrc&productGroup=96 was digested with various restriction enzymes, purified and treated with T4 DNA ligase to circularise fragments. The DNA was PCR amplified using two (‘outward-facing’) primers expected to give no product on linear DNA, XR6 and XF2, which resulted in ~ 1.8 and ~ 2.8 kbp DNAs when BAC 28C10 DNA had been digested with SacII and Xhol respectively. The PCR fragments were sequenced using XR1 and additional primers.

Genomic DNA of pacX mutants was PCR amplified using pacX-specific primers (e.g., XF2 and XR8), and the fragments were sequenced using additional gene specific primers. Mutations were, in most cases, confirmed on the opposite strand of a different PCR fragment.

Primer sequences are given in Table S1.

In silico analyses

PacX orthologues were searched in the JGI (http://genome.jgi-psf.org/programs/fungi/index.jsf) and NCBI databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A. nidulans FotI-like elements were searched in http://www.aspgd.org/ (Cerqueira et al., 2014). Alignments were carried out with MAFFT version 7.5, http://mafft.cbrc.jp/alignment/server/ (Katoh and Standley, 2013), refinement with BMGE, http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::BMGE (Criscuolo and Gribaldo, 2010), Maximum Likelihood Phylogeny with PhyML, http://phylogeny.lirmm.fr/phylo_cgi/alonec.py (Dereeper et al., 2008; Guindon et al., 2010) calculating also approximate likelihood tests (Anisimova and Gascuel, 2006). Tree drawing was done with Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Colled-coil prediction was carried out with http://toolkit.tuebingen.mpg.de/pc coils (Parry, 1982; Lupas et al., 1991; Lupas, 1996). Conservation and putative functionality of individual PacX residues was calculated with ConSurf, http://consurf.tau.ac.il/ (Ashkenazy et al., 2010; Celniker et al., 2013). Nuclear localisation signals were searched with nLoc in http://genome.unmc.edu/nLoc/index.html (King and Guda, 2007) PSORT II, http://psort.hgc.jp/form2.html (Nakao and Nakai, 2002), cNLS Mapper, http://nls.mapper.ib. keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi (Kosugi et al., 2009) NetNes, http://www.cbs.dtu.dk/services/NetNES/ (La Cour et al., 2004).

Microscopy and imaging techniques

For PacX localisation studies, the PacX::GFP HhoA::mCherry strain ALO2 was cultured at 28°C in LabTek chambers (Roch-
ester NY) containing WMM [watch minimal medium (Peñalva, 2005)], adjusted to acid or alkaline pH as described (Galindo et al., 2007). Epifluorescence images were acquired with a Leica DMI6000 inverted optics microscope coupled to a Hamamatsu ORCA ERII camera, using Metamorph software (Molecular Devices) and SemrockBrightlinefilter sets for red and green fluorescence emission, essentially as described (Pantazopoulou and Peñalva, 2009; 2011). Z-stacks of images were processed using the Metamorph ‘unsharp’ filter and, when needed, used to construct maximal intensity projections. Images were exported to Corel as TIFF maps.

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