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Review

Gene targeting in filamentous fungi: the benefits of impaired repair

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ABSTRACT

The extent of published fungal genome sequences call for sophisticated genetic tools to address the functions and features of annotated gene products. Gene targeting is the method of choice to assess the cellular role of a given protein, trying to replace the coding sequence by selectable markers or to fuse it to functional modules, e. g. fluorescent labels. This approach relies on homologous integration of exogenous DNA fragments, the rate of which is determined by two competing processes of double strand break repair: illegitimate and homologous recombination. Advances in targeting fungal genes have recently been achieved by suppression of the former process; for several filamentous fungi a dramatic increase in homologous integration rates is evident when this so-called non-homologous end joining pathway is inoperative, which opens the prospect of comprehensive functional studies at high throughput.

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1. Introduction

The precise manipulation of genes is a key approach in fungal molecular biology. Inactivation or replacement of a given locus hints towards a cellular role of its product, and therefore sophisticated means of gene targeting have been developed to support functional methodologies in fungal research. The basic experimental setup is that of transforming the respective organism with a suitable DNA fragment to delete, replace or disrupt the target locus, an incident that has to be selectable on an appropriate type of medium. In case the DNA fragment contains no functional elements for autonomous replication or segregation, it is either degraded or, after integration, maintained as part of the fungal genome. Basically, two mechanisms of DNA double strand repair ensure that an introduced piece of DNA is pasted into the fungal genome to be stable replicated: homologous recombination (HR) and non-homologous end joining (NHEJ), also called illegitimate recombination. The former requires stretches of homologous or homeologous sequence, while the latter joins DNA ends without homology. With some exceptions including the model eukaryote *Saccharomyces cerevisiae*, fungi appear to favour NHEJ over HR resulting in low gene targeting efficiencies, making precise genome manipulations tedious and often time-consuming. Recent experimental approaches have successfully overcome this obstacle for a variety of fungal organisms, and this *Technical Focus* aims to summarize these latest developments in fungal molecular biology briefly.

2. NHEJ vs. HR

Organisms commonly have to deal with free DNA ends as they are generated by exogenous agents or during cellular

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recombination events. To ensure genome integrity, distinct cellular machineries have evolved that act on such double strand breaks (DSB), either homology-directed or *via* direct joining (Paques & Haber 1999). In molecular biology, the manipulation of a gene of interest is often achieved by integration of an extra-chromosomal DNA fragment into a target site within the genome, a step that is mediated by homologous sequence stretches and therefore relies on HR.

The RAD52 gene group that comprises several components of the cellular HR machinery has been characterised at the molecular level (Symington 2002), mainly based on research in the baker's yeast *S. cerevisiae* in which HR is extremely efficient and therefore the principal way of foreign DNA integration. In contrast to this, the non-homologous or illegitimate repair pathway has evolved as the predominant one in most filamentous fungi as well as in higher eukaryotes such as plants and mammals, maybe due to the abundance of repetitive sequences in their genomes. A large body of knowledge has accumulated to elucidate the precise orchestration of this repair procedure (Critchlow & Jackson 1998; Daley *et al.* 2005; Hefferin & Tomkinson 2005).

Based on findings in mammalian cell lines or microorganisms, the current model of the NHEJ mechanism involves several basic steps: binding of proteins to the free DNA ends and bridging them, which is accompanied by constitution of a protein kinase as well as a ligase activity, processing and gap filling, and eventually ligation of the lesion. Pivotal for the first step is a heterodimeric protein complex termed Ku that acts as a DNA end-binding factor (Featherstone & Jackson 1999). Its subunits, the Ku70 and Ku80 components, comprise a conserved core that is flanked by N- and C-terminal domains, which presumably mediate interactions with additional partners of the NHEJ pathway. Solving the three-dimensional structure of the mammalian Ku complex revealed its toroidal shape that contains positively charged amino acids inside an asymmetrical ring (Walker *et al.* 2001). Accordingly, the Ku complex binds to the ends of double-stranded DNA in a non-specific fashion to act as a platform for the subsequent recruitment of protein complexes, such as the catalytic subunit of the DNA-dependent kinase, DNA-PK$_{cs}$, and the XRCC4-DNA ligase IV complex (Jeggo 1998).

The current data on double strand repair indicate that the outcome of a competition between Rad52 and the Ku heterodimer in binding free DNA ends determines the mode of integration of extra-chromosomal DNA segments (Haber 1999). Depending on which 'gatekeeper of recombination' binds to the ends of an introduced DNA fragment, gene targeting *via* HR or ectopic integration by NHEJ, respectively, occurs (Fig. 1).

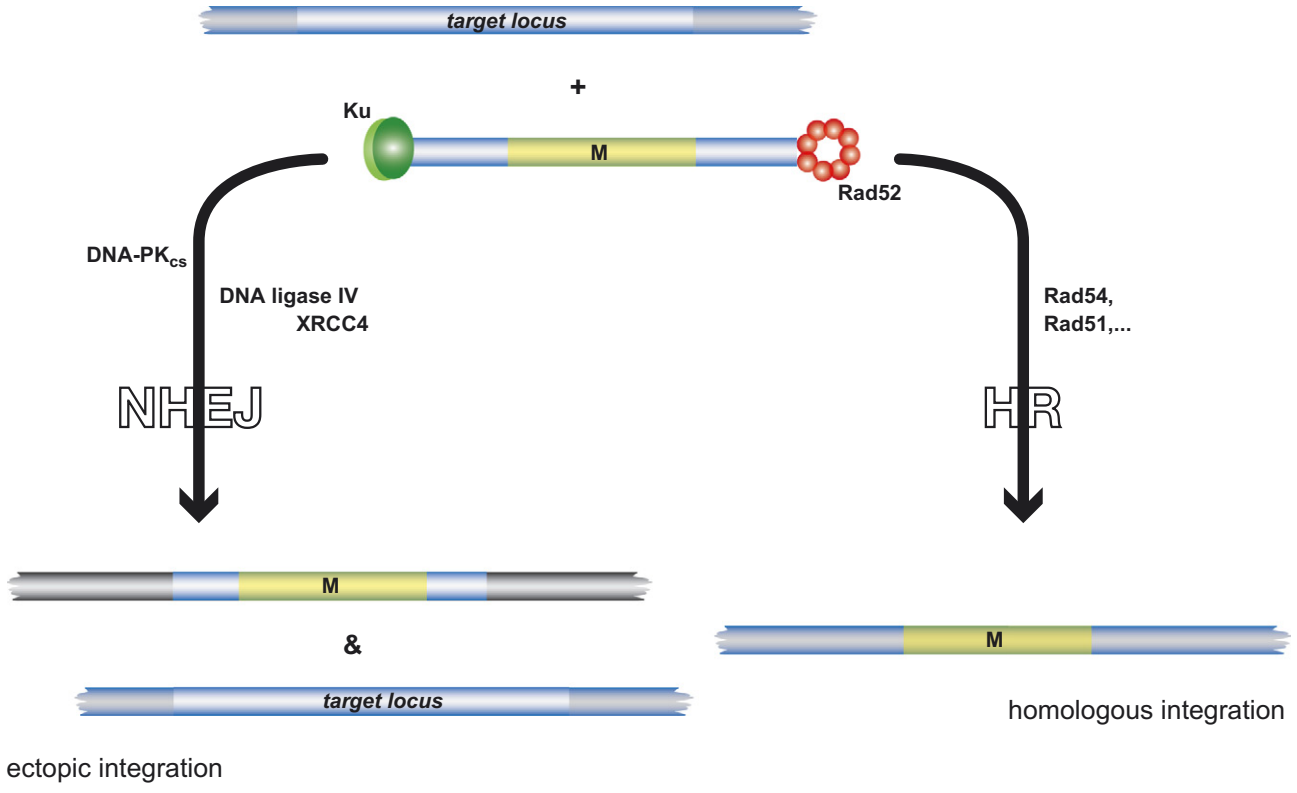

Fig. 1 – Gatekeepers of recombination determine the fate of exogenous DNA in fungi. Binding of protein complexes to its free ends decides on the mode of integration of a hosted DNA fragment: attachment of the heterodimeric Ku complex initiates recruitment of the DNA-PK$_{cs}$ subunit together with ligase IV/XRCC activity to facilitate non-homologous end joining (NHEJ) of DNA ends, interaction with Rad52 and additional recombination proteins promotes homologous recombination (HR) and accordingly gene conversion. Whereas the former process eventually results in ectopic integration of a replacement module (M) into the recipient's genome, the latter is crucial to achieve targeted gene replacement.

3. Impaired NHEJ boosts gene targeting in fungi

Ectopic integration is the major fate of transformed DNA in most fungi, based on the fact that NHEJ is more active or that HR is less efficient. Accordingly, long stretches of homologous sequence, spanning several thousand base pairs, have to be incorporated in gene targeting constructs to achieve feasible rates of HR and gene targeting. Although sophisticated approaches, for instance recombineering, are available to assemble such replacement modules, their handling and downstream processing remains tedious (Chaveroche *et al.* 2000; Court *et al.* 2002). Moreover, targeting efficiencies as observed in *S. cerevisiae*, where homology arms shorter than 100 bp still yield high targeting efficiencies close to 100 %, are not even achieved with these cassettes; accordingly, a large fraction of transformants needs to be screened for the desired targeting event, which is often a laborious and time-consuming task. Consequently, two alternative strategies can be followed to make fungal gene targeting more efficient: enhancing HR or impairing NHEJ.

The latter approach has proven to be highly beneficial in several fungi by generating mutants that lack components of the Ku complex (Table 1). The first data on successfully improving gene targeting by impairing the NHEJ pathway stems from work on the budding yeast *Kluyveromyces lactis* (Kooistra *et al.* 2004). There, deletion of the *KlKU80* gene increases targeting efficiencies dramatically, even for homologous flanking regions in the 100 bp-range. However, reducing the spans of target gene homology resulted in decreased transformation efficiencies, which was more pronounced for the *KlKU80Δ* mutant in comparison to its *wild-type* progenitor.

Shortly after this report, the first evaluation of gene targeting in an NHEJ-deficient filamentous fungus was made public: homologues of both Ku subunits in the ascomycete *Neurospora crassa* could be identified, and targeting of two genes was assessed in corresponding *mus-51* (Ku70) and *mus-52* (Ku80) deletion mutants (Ninomiya *et al.* 2004). Whereas a *N. crassa wild-type* strain typically shows levels of homologous gene replacement between 5 and 20 %, for both NHEJ$^-$ mutants a significant increase of homologous integration events is evident: homology arms of 500 bp and 1000 bp resulted in 90 % and 100 % of correct transformants, respectively. A more detailed inspection of these mutant strains revealed additional phenotypes, such as mildly increased sensitivity towards chemical agents like methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), and bleomycin, which is in line with the cellular role of the NHEJ pathway in DSB repair. In a recent report, more data on the different ways of foreign DNA integration in *N. crassa* were obtained (Ishibashi *et al.* 2006). Strikingly, deletion of the *LIG4* orthologue *mus-53*, which encodes a key regulator of non-homologous integration, resulted in exclusive homologous integration even when short homology arms of 100 bp were used. However, this phenotype is accompanied by a pronounced decrease in transformation frequencies and again sensitivity towards mutagens.

Based on these findings in *N. crassa*, a couple of follow-up reports on other fungi addressing the rate of homologous

Table 1 – Homologous integration rates of fungal mutants deficient in NHEJ

Fungal Organism	Genotype	HIF[a] (%)	Flanks[b] (bp)	Reference
Aspergillus fumigatus	*wild-type*	5–10	1000	
	akuAΔ (ku70)	96	1000	Krappmann *et al.* 2006
	akuBΔ (ku80)	80	1500	da Silva Ferreira *et al.* 2006
Aspergillus nidulans	*wild-type*	38	500	
	nkuAΔ (ku70)	89	500	Nayak *et al.* 2006
Aspergillus oryzae	*wild-type*	11	~1400	
	ku70Δ	63	~1400	Takahashi *et al.* 2006b
Aspergillus sojae	*wild-type*	1.4	~1400	
	ku70Δ	75	~1400	
	ku80Δ	64	~1400	Takahashi *et al.* 2006b
Cryptococcus neoformans	*wild-type*	~65/20–50	900/450	
	cku70Δ	100/91	900/450	
	cku80Δ	~95/100	900/450	Goins *et al.* 2006
Kluyveromyces lactis	*wild-type*	80/4	500/50	
	KlKU80Δ	100/100	500/50	Kooistra *et al.* 2004
Neurospora crassa	*wild-type*	21/9/2	1000/500/100	
	mus-51Δ (ku70)	100/91/10	1000/500/100	
	mus-52Δ (ku80)	100/93/4	1000/500/100	Ninomiya *et al.* 2004
	wild-type	23	2000	
	mus-53Δ (lig4)	100	2000	Ishibashi *et al.* 2006
Sordaria macrospora	*wild-type*	2.5–4.1	~1100	
	ku70Δ	>85	~1100	Pöggeler & Kück 2006

a Homologous integration frequency: ratio of correct integrants to total transformants × 100.
b Length of homology arms in gene targeting cassettes.

integration in NHEJ-deficient genetic backgrounds were published, such as for the human pathogens *Aspergillus fumigatus* or *Cryptococcus neoformans*, the model organism *Aspergillus nidulans*, the *koji* molds *Aspergillus oryzae* and *Aspergillus sojae*, and the filamentous ascomycete *Sordaria macrospora* (Table 1).

The most comprehensive evaluation of the system was performed for *A. nidulans*, in which both Ku-encoding genes, *nkuA* and *nkuB*, were deleted to assess phenotypical appearance and gene targeting capacities of the corresponding mutant strains (Nayak *et al.* 2006). All Ku-deficient isolates grew with rates similar to their *wild-type* progenitor, and furthermore, no markedly increased sensitivity towards DNA damaging agents like MMS, bleomycin, or camptothecin could be detected. Yet, relative frequencies of homologous integration were significantly increased as determined in replacement experiments for homokarya or heterokarya, with flanking regions of 500 bp being sufficient to yield a proportion of correct transformants of almost 90 %. Paralleling this study, the genes encoding the Ku complex subunits in the biotechnologically important aspergilli *A. sojae* and *A. oryzae* were identified and disrupted to generate NHEJ-deficient downstream recipients (Takahashi *et al.* 2006a, b). Phenotypic characterisations revealed no hypersensitivity towards the DNA damaging agents MMS and phleomycin, and targeting frequencies were reliably high when replacement cassettes with homology arms in the kbp-range were transformed. By shortening the length of these flanks the transformation rates dropped, accompanied by a decrease in targeting efficiencies, which illustrates a common pattern for most fungi impaired in NHEJ.

Two concomitant studies have addressed the targeting efficiency of Ku-ablated *A. fumigatus* strains with comparable results (da Silva Ferreira *et al.* 2006; Krappmann *et al.* 2006): either Ku70- (*akuA⊿*) or Ku80- (*akuB⊿*) deficiency results in dramatically increased gene targeting frequencies but no additional obvious phenotypes except increased MMS sensitivity. Notably, in a murine low-dose infection model of pulmonary aspergillosis the *akuB⊿* strain was tested as virulent as a control strain resembling a *wild-type* isolate of this opportunistic pathogen, a finding that was substantiated in an alternative virulence test system for the *akuA⊿* strain.

This important phenotypical behaviour could also be observed for the fungal yeast pathogen *C. neoformans* in competitive systemic infection experiments when probing a *cku70⊿* strain or a *cku70⊿cku80* double mutant in mice (Goins *et al.* 2006). Besides this, homologous integration rates of gene replacement cassettes were greatly increased to levels between 90 and 100 % with homologous flanking sequences of about 450 bp and 900 bp, respectively. Apart from an increased sensitivity to the antibiotic phleomycin, no discernable phenotypes with respect to DNA damaging agents were detected. In line with the *wild-type*-like virulence of *C. neoformans cku⊿* mutants, no alterations for two distinct pathogenicity determinants – melanin production and capsule formation – were observed.

As the most recent member in the family of NHEJ-deficient fungi, the ascomycete *Sordaria macrospora* has been described, which serves as model system for fungal sexual differentiation and is also characterised by poor rates of homologous integration. Similar to other filamentous fungi, deletion of a Ku gene increases targeting efficiencies drastically, as exemplified for two gene loci (Pöggeler & Kück 2006): whereas the *wild-type* strain displays homologous integration rates below 5 % when using replacement cassettes with homology arms in the range of 1000 bp, efficient gene targeting could be determined for a congenic *ku70⊿* strain with rates better than 85 %, yet accompanied by significantly less transformants. No differences could be detected between the *S. macrospora wild-type* isolate and its *ku70⊿* derivative with respect to vegetative growth and sexual development, which allows easy elimination of the *ku70⊿* mutation by crossing and progeny analysis.

4. The pros and cons of NHEJ-deficient mutants

The most obvious application of fungal strains deficient in the NHEJ branch of DNA repair lies in their high relative rates of correct gene targeting to overcome high backgrounds of ectopic integration. Gene targeting includes genetic manipulations such as gene disruption, promoter replacement, or fusing the coding sequence to suitable tag modules; all these molecular techniques help to determine cellular functions of given gene products as they are disclosed in abundance by genome annotation projects. NHEJ-deficient recipient strains thus strengthen our armoury for functional genomic studies. In the model ascomycete *A. nidulans* this versatility could be demonstrated for sophisticated approaches such as gene replacement/heterokaryon rescue to determine the phenotypes of lethal gene lesions (Osmani *et al.* 2006). Comprehensive gene deletions at high throughput as commenced for the ascomycete *N. crassa* are now achievable by the use of a Ku^{-} recipient strain, a project pivotal for the development of large-scale functional studies in filamentous fungi (Colot *et al.* 2006). An obvious shortcoming of the $NHEJ^{-}$ background in such systematic gene deletion collections lies in possible synthetic phenotypes.

The group of filamentous fungi for which NHEJ-deficient mutant strains has been generated is about to expand, accompanied by an accumulation of phenotypical information. Reliable data on unwanted yet undiscovered side effects that might be linked to interference with the NHEJ branch of double strand break repair are scarce. Consequently, it is of great importance to assess corresponding mutant strains with respect to genetic stability or genome integrity, as phenotypes might appear after prolonged cultivation over several generations. This view is strengthened by the impact of the yeast Ku complex on such fundamental cellular aspects as telomere maintenance, nuclear spatial organisation, or mitotic recombination (Mages *et al.* 1996; Boulton & Jackson 1998; Taddei *et al.* 2004). By crossing out the genetic lesion, this potential drawback can be minimized in sexually propagating fungi. For deuteromycetes however, other and more tedious approaches need to be developed to ensure transient inactivation of the Ku complex; this is considered necessary especially for medically relevant fungi, as downstream analyses such as virulence tests in animals must not be biased by uncontrolled genetic side effects that may be supported by impairment of the NHEJ pathway.

In conclusion, fungal $NHEJ^{-}$ recipients provide the basis for functional studies at large and broad scale and they will indisputably serve as powerful instruments in the post-genomic era of fungal molecular biology.

5. Note Added in Proof

In the course of issuing this review, data on NHEJ-deficient strains of the biotechnological workhorse *Aspergillus niger* were made public. In the relevant article, gene targeting efficiencies in a *kusA* (*ku70*) deletion mutant are described to reach over 80% when homology regions of 500 bp are used, in contrast to a *wild-type* recipient for which 7% were determined (Meyer *et al.* 2007). Accordingly, high frequencies of heterokaryon formation in a group of primary transformants were observed, which is the basis for the so-called heterokaryon rescue approach to characterise essential genes (Osmani *et al.* 2006). Phenotypically, the *kusA⊿* mutant displayed no obvious growth defect but appeared sensitive towards X-ray and UV exposure. With the recently disclosed genome sequence of this filamentous fungus (Pel *et al.* 2007), NHEJ-deficient *A. niger* recipients will facilitate functional genomics studies to support fungal biotechnology.

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