Extending the *Schizosaccharomyces pombe* Molecular Genetic Toolbox

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Abstract

Targeted alteration of the genome lies at the heart of the exploitation of *S. pombe* as a model system. The rate of analysis is often determined by the efficiency with which a target locus can be manipulated. For most loci this is not a problem, however for some loci, such as fin1⁰, rates of gene targeting below 5% can limit the scope and scale of manipulations that are feasible within a reasonable time frame. We now describe a simple modification of transformation procedure for directintgration of genomic sequences that leads to a 5-fold increase in the transformation efficiency when antibiotic based dominant selection markers are used. We also show that removal of the pku70⁰ and pku80⁰ genes, which encode DNA end binding proteins required for the non-homologous end joining DNA repair pathway, increases the efficiency of gene targeting at fin1⁰ to around 75–80% (a 16-fold increase). We describe how a natMX⁶/rpil42⁰ cassette can be used for positive and negative selection for integration at a targeted locus. To facilitate the evaluation of the impact of a series of mutations on the function of a gene of interest we have generated three vector series that rely upon different selectable markers to direct the expression of tagged/untagged molecules from distinct genomic integration sites. pINTL and pINTK vectors use ura⁴ selection to direct disruptive integration of leu¹⁰ and lys¹⁰ respectively, while pINTH vectors exploit nourseothricin resistance to detect the targeted disruption of a hygromycin B resistance conferring hphMX⁶ cassette that has been integrated on chromosome III. Finally, we have generated a series of multi-copy expression vectors that use resistance to nourseothricin or kanamycin/G418 to select for propagation in prototrophic hosts. Collectively these protocol modifications and vectors extend the versatility of this key model system.

Introduction

The genetic malleability of the fission yeast *S. pombe* has helped it to maintain a prominent position alongside the more extensively exploited budding yeast *Saccharomyces cerevisiae*, as a powerful model system for the characterisation of the basic facets of eukaryotic cell and molecular biology. This malleability is based upon an extensive repertoire of classical and molecular genetic techniques [1,2,3]. As in budding yeast these techniques were initially based upon the exploitation of key auxotrophic markers.

Classical genetic analysis the adenine biosynthesis pathway in *S. pombe* highlighted the utility of the colony-colour change resulting from the accumulation of P-ribosylaminoimidazole in ade6 mutants that is then oxidised to a red pigment [4]. The ability to use this red pigmentation as a reporter for Ade6 function made this locus a major focus for studies of core genetic principles. These studies led to the development of a number of useful genetic tools including ade6M210/ade6M216 hetero-allelic complementation for the selection and maintenance of diploid strains [5] and the use of the sup3.5 opal suppressor tRNAser mutation as a marker for selection in an ade6.704 mutant background [6,7,8]. Cross species complementation of *S. pombe* ade6 mutations with the *S. cerevisiae* LEU2⁰ gene was initially used to apply existing budding yeast technology to fission yeast [9], but remains a widely used selectable
marker to this day because the lack of homology to sequences in the S. pombe genome means that it does not direct integration into a specific genomic site. However, when used as a marker to select for site specific integration, multiple integration events can occur [10], suggesting either that the heterologous expression of the LEU2 gene is barely sufficient for growth at low copy number or that the budding yeast enzyme is less attuned to fission yeast physiology than the native 3-isopropyl malate dehydrogenase enzyme, Leu1. Transposition of the lessons learnt from the exploitation of the budding yeast ornithine decarboxylase CAD2 gene for positive and negative selection [11] led to the deletion of the adefu gene from S. pombe to generate the adefu allele that is so widely used in the field today [12] with many adefu based vectors [13,14,15,16]. Continued developments are considerably expanding the array of available auxotrophy-complementing markers to include: adefu, hisi, his2, his3, his5, arg3, arg12, lys1, lys2 and tyr1 [17,10,19,20,21,22,23,24]. However, his3+, LEU2 and adefu remain the most widely-used markers for selection of multi-copy vectors in common use. Integration vectors that target a particular heterologous locus have been less extensively developed, however the pDUAL series and pJK148 vectors are used widely as they exploit recombination to convert the leucine auxotrophy of leu1.32 to leucine prototrophy to select integration at the leu1 locus [25,26,27]. The pJK210 uses a similar rescue of ura4.294 to target integration at the leu1 locus [25].

While these auxotrophic selection markers offer powerful tools, they also create the need to introduce an increasingly complex array of background markers into a strain of interest. Not only is this time consuming but many combinations of deficiencies in amino acid provision compromise a host strain’s fitness on certain media, which may complicate the interpretation of the phenotype arising from the mutation of interest. Furthermore, the sensitivity of the broadly acting TOR signalling network to addition of leucine to the medium [28] indicates that provision of amino acids demanded by the use of auxotrophic markers and perhaps the auxotrophic markers themselves are not merely passive players in cellular homeostasis, but can influence the control networks that impinge upon diverse processes from metabolism, through cell cycle control, sexual differentiation, and the actin cytoskeleton. Thus, controlling the genetic context within which the consequences of particular mutations are studied in prototrophs not only accelerates the rate of analysis, but avoids both anticipated and unforeseen complications arising from interplay between pathways.

Following the highly successful exploitation of antibiotic resistance genes as dominant selectable markers for PCR based tagging and deletion approaches in the budding yeast S. cerevisiae [29,30,31,32,33,34], the technology has been adapted for use in a variety of fungi including S. pombe. Genes conferring resistance to kanamycin/G418, hygromycin B, phleomycin/bleomycin and nourseothricin/ClonNat are highly effective dominant markers in fission yeast [35,36,37,38]. They have been extensively exploited in an increasing array of “PCR tagging vectors” in which oligo-nucleotides, that fuse vector sequences to short oligo-nucleotides, that fuse vector sequences to short fragments of the genomic DNA, are added to generate final concentrations of 100 μg/ml in the growth medium where appropriate.

Transformation of S. pombe

Cells were grown to mid-log phase in YES (4x10⁶ cells/ml). After harvesting cells were washed with H₂O, 0.1 M Lithium Acetate (pH 4.9) and resuspended in 0.1 M Lithium Acetate (pH 4.9) at 10⁶ cells ml⁻¹. After 1 h incubation at 23°C 1-5 μg DNA and 290 μl of 50% PEG4000 (freshly made in sterile 0.1 M Lithium Acetate, pH 4.9 for each transformation) was added to generate final concentrations of 100 μg/ml in the growth medium where appropriate.

Molecular Genetics

Generating pINTL vectors. A 686 bp fragment of pUC19, including the MCS and NdeI site, were removed by Phusion mediated deletion (New England Biolabs) using oligo nucleotides BH1 and BH2, creating a NotI site. A 2.32 kb fragment extending
from −456 to +752 of the *S. pombe* *leu1*+ gene was amplified as a *Nol* fragment using oligo-nucleotides BH3 and BH4 and cloned into the modified pUC19 vector to create pINT1. An 1.78 kb fragment extending from −516 to +1186 of the *S. pombe* *ura4*+ gene was amplified from pURA4 [12] using oligonucleotides BH5 and BH6 to flank the *ura4*+ sequences with 5′ *PstI* site and 3′ *SacI* sites. Oligos BH7 and BH8 were used to amplify pINT1, which was then used as recipient for the amplified *ura4*+ fragment within the *leu1*+ open reading frame by Gibson-mediated integration (New England Biolabs) to generate pINTLA that can act as recipient for any *PstI/SacI* fragment containing promoter-insert-terminator (Figure S1). The remaining plasmids in the pINTL series were generated by cloning the appropriate *PstI/SacI* fragment from a relevant *pREP* tagging vector [16]. pINTL41PkN was generated by insertion of the *PstI/SacI* fragment of *pREP41PkN* into pINTLA followed by the *SacI/SacI* fragment from *pREP41PkN*. Full sequences of the pINTL vectors are presented in Figure S1.

**Generating pINTK vectors.** The *lys1* 5′ region was amplified (VS642/VS644) to introduce *HindIII NotI* sites at one end and *PstI* site at the other end. The *lys1* 3′ region was amplified (VS645/VS646) to introduce a *KpnI* site at one end and *EcoRI NotI* sites at the other end. Both fragments were cloned *HindIII - PstI* and *KpnI - EcoRI*, respectively into pGEM3. The *loxP-ura4* cassette was generated by PCR amplification of the *ura4*+ gene (VS647/VS648) to introduce *KpnI* site and a LoxP site on one end and *SmaI SacI* sites with the LoxP site on the other end. This fragment was then cloned as a *KpnI - SmaI* fragment into the *lys1*+ containing vector to generate pINTK, pINTK81, pINTK41 and pINTK1 by

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**Figure 1. Manipulating native loci with an rpl42*+/natMX6* cassette. A) Approaches used for targeted mutagenesis. B) The structure of the pFA6arp42natMX6 plasmid. C) The phenotype switches arising from the progression through the indicated genotypes.

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cloning the nmt promoters from pREP81, pREP41 and pREP1 respectively as PstI – BamHI fragments into pINTK. GFP, CFP and YFP tags were amplified to introduce a BamHI – SacI cloning site at their termini before being cloned into the pINTK vector to generate pINTK1-6His. The sequence of the complementary oligonucleotides (VS1482/VS1483) into the pINTH vector was Quickchange (Stratagene) silent mutation to remove the nmt promoters from pREP81, pREP41 and pREP1 based plasmids were then inserted into the pINT** vector series. Because the multi-cloning site of pREP41PKN and pREP81PKN contains a SacI site, the pINTH1PKN and pINTH81PKN were generated by sequential insertion of the appropriate SacI and PstI-SacI fragments from pREP41PKN and pREP81PKN respectively, pINT* was digested with NdeI and KpnI, end filled with Klenow polymerase (New England Biolabs) to remove a 189 bp fragment and re-ligated to remove the NdeI site of pUC19 to generate pINT**. PstI SacI sequences containing the nmt promoter - cloning site/tag - nmt terminator cassettes from pREP1, pREP41 and pREP81 based plasmids were then inserted between PstI SacI sites in pINT** to generate the pINT vector. The first step in the generation of the pINT vector was Quickchange (Stratagene) silent mutagenesis to remove the hphMX6 (CTGCAG>CCTGCA and CATATG>CATTGG, respectively) and a XmnI/SmaI site from natMX6 (CCCGGG>CCCAGG). The following fragments were amplified using the indicated primers to introduce restriction sites at their termini before being cloned into the vector ZeroBluntTOPO (Invitrogen): one 0.85 kb half of hphMX6 flanked with PstI and NotI HindIII (primers DF1 and DF2); the remaining 0.85 kb fragment flanked by EcoRI and EcoRI NotI (primers DF3 and DF4); natMX6 flanked with SacI and EcoRI (primers DF5 and DF6). The SacI – EcoRI natMX6 fragment was inserted into pUC19 followed by the EcoRI and PstI–HindIII hphMX6 fragments to generate pINT*. pINT* was digested PstI and KpnI, end filled with Klenow polymerase (New England Biolabs) to remove a 189 bp fragment and re-ligated to generate the NdeI site of pUC19 to generate pINT**. PstI SacI sequences containing the nmt promoter - cloning site/tag - nmt terminator cassettes from pREP1, pREP41 and pREP81 based plasmids were then inserted between PstI SacI sites in pINT** to generate the vector series. Because the multi-cloning site of pREP41PKN and pREP81PKN contains a SacI site, the pINTH1PKN and pINTH81PKN were generated by sequential insertion of the appropriate SacI and PstI-SacI fragments from pREP41PKN and pREP81PKN respectively, pINT* was digested with NdeI, end filled with Klenow polymerase (New England Biolabs) and re-ligated to

### Table 1. Transformation efficiencies.

| Transformation | 10⁶ cells transformed with 1 μg DNA for the indicated transformation |
|---------------|--------------------------------------------------------------------------------|
|               | ura4 into leu1 | kanMX6 into pku80 | natMX6 into pku80 | hphMX6 into pku80 | bleMX6 into pku80 |
| Spread directly to selective media | 950 | 0 | 0 | 0 | 0 |
| Spread to YES, replica plate to selective media after 20 h | 1000 | 150 | 140 | 120 | 100 |
| Incubate in MSL for 2 h, spread to selective media | 900 | 0 | 0 | 0 | 0 |
| Incubate in MSL for 4 h, spread to selective media | 950 | 3 | 2 | 0 | 1 |
| Incubate in MSL for 8 h, spread to selective media | 1000 | 4 | 5 | 3 | 3 |
| Incubate in MSL for 16 h, spread to selective media | 1100 | 650 | 700 | 600 | 650 |
| Incubate in MSL for 20 h, spread to selective media | 1100 | 850 | 800 | 750 | 800 |

The table shows the number of transformants obtained when 1 μg of the indicated DNA fragments was transformed into identical numbers of competent cells of the indicated strains. Each transformation mix was split into seven equal aliquots that were treated as indicated in the column on the left.

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### Table 2. Strains used in this study.

| Lab Strain number | Genotype | YGRC strain number | Source |
|-------------------|----------|--------------------|--------|
| IH5974            | 972 h    | Lab stock          |        |
| IH1308            | ura4D18 h    | Lab stock          |        |
| IH8794            | rp42+p56Q leu1.32 ura4D18 h   | Rougve et al. 2007  |        |
| IH5221            | pku70-his3 leu1.32 his3.D1 ade6.M216 ura4.d18 h    | FY23684 | Lab stock |
| IH6067            | pku70-natMX6 leu1.32 ura4D18 his2 h    | FY23686 | Manolis et al. 2001 |
| IH12994           | pku70-natMX6 ura4D18  | FY23687 | This study |
| IH12959           | pku70-hphMX6′ leu1.32 ura4D18 his2 h    | FY23685 | This study |
| IH6114            | pku80-ura4′ leu1.32 ura4D18 his2 h    | FY23691 | Manolis et al. 2001 |
| IH13006           | pku80-natMX6  | FY23689 | This study |
| IH12958           | pku80-natMX6′ leu1.32 ura4D18  | FY23690 | This study |
| IH12960           | pku80-hphMX6 ura4D18 his2 h    | FY23688 | This study |
| IH5869            | hph171k h    | FY23692 | This study |
| IH6365            | leu1::nmt41fin1.KD-pknur4+ ura4D18  | This study |
| IH6366            | leu1::nmt81fin1.KD-pknur4+ ura4D18  | This study |
| IH6364            | hph171k::nmt41fin1.KD-pknur4+ natMX6 ura4D18  | This study |
| IH6409            | hph171k::nmt81fin1.KD-pknur4+ natMX6 ura4D18  | This study |

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Figure 2. Inclusion of *pku70* and *pku80* in host strain radically enhances targeting at the *fin1* locus. A) Cartoons depicting the structure of the DNA fragments used to direct the integration of a *natMX6* cassette 3’ to the Fin1 coding sequences at the *fin1* locus and the integration of sequences encoding three GFP molecules, a stop codon and the *kanRMX6* marker at the end of the *fin1* locus. B) PCR amplification.
generate pINTHA. Full sequences of the pINTH vectors are presented in Figure S3.

**Generating pREPN and pREPK vectors.** To generate the pREPN vectors, the Smal sites of the natMX6 gene in natMX6 cassette were destroyed (the NdeI site in pFA6anatMX6 is outside of the cassette). The natMX6 gene was amplified with the oligonucleotides AG1 and AG2 that had 20 nucleotides homology to the ends of the natMX6 cassette and 80 bp homology with the sequences adjacent to the LEU2 integration site of pREPI. 5 μg of the natMX6 fragment was transformed alongside 1 μg of the appropriate LEU2 based pREP vector that had been linearised by digestion with KpnI that cut inside the LEU2 marker gene of the relevant vector in host IH147. Plasmids were isolated from two antibiotic resistant leucine auxotrophic colonies with the DNA selected for by placing transformants generated an 850 bp fragment in the recipient host. For the ''transformation amplification with the same primers used to screen ''fin1 ORF'' transformants generated an 850 bp fragment in the correct transformant (red tick next to each panel). C) A table showing the frequency of correct integration events in the indicated strains with the indicated transformants generated an 850 bp fragment in the recipient host (red cross next to each panel) and an 4650 bp fragment in the correct transformant (red tick next to each panel). For the ``fin1.3GFP'' transformation amplification with the same primers used to screen ``fin1 ORF'' transformants generated an 850 bp fragment in the recipient host (red cross next to each panel) and an 4650 bp fragment in the correct transformant (red tick next to each panel). For the ``transformation amplification with the same primers used to screen'' transformants generated an 850 bp fragment in the recipient host (red cross next to each panel) and an 4650 bp fragment in the correct transformant (red tick next to each panel). For the ``transformation amplification with the same primers used to screen'' transformants generated an 850 bp fragment in the recipient host (red cross next to each panel) and an 4650 bp fragment in the correct transformant (red tick next to each panel).

**Results**

**A natMX6/rpl42 Cassette for Positive and Negative Selection**

To study the significance of phosphorylation events in the timing and execution of cell division we mutate candidate sites at endogenous loci [60,61,62]. We first integrate a marker at the gene of interest (goi) before transforming this new host strain with a fragment whose homology to the genome extends beyond either side of the integration site. As this fragment harbours a goi mutation, positively selecting for marker loss and screening by DNA sequencing identifies the candidate with the desired mutation (Figure 1A). Although the ability to apply both positive and negative selection for ura4+ make it an ideal marker for this purpose [12], the cost of FOA can become limiting in a programme that targets multiple mutants to multiple loci. Similarly, the need to express human equilibrative nucleoside transporter, hENT1, to use thymidine analogues for positive selection [63] limits the appeal of this alternative approach. To generate a rapid and cheap alternative to these two options we have combined the strong positive selection of natMX6 [58] with the rpl42 recessive cycloheximide resistance marker system developed by Krogan and colleagues [57] in a natMX6/rpl42 double cassette in pFA6arpl42natMX6 (Figure 1B). Resistance to nourseothricin selects for integration of this cassette in an rpl42::P56Q host strain. Subsequent replacement of the cassette by an overlapping sequence is selected for by placing transforms onto plates containing cycloheximide (Figure 1C).

**Overnight MSL-N Incubation Enhances Transformation Efficiency**

Our ability to manipulate native loci has been confounded by varying efficiencies of targeted integration. For some loci, such as fin1+, targeted integration was uncommon, with around a 5% chance that a transformant was the desired integration event, prompting us to seek strategies that may improve transformation and targeting efficiencies.

When using antibiotic selection for integrative transformation, transformants are incubated in non-selective conditions for 18 hours before applying selection to enable them to accumulate sufficient enzyme from the newly generated expression cassette to survive the otherwise lethal impact of the antibiotic [40]. Traditionally this “recovery phase” has been applied by spreading cells on non-selective plates before replica plating the ensuing lawn of cells onto antibiotic containing plates 18 hours later [40]. While highly effective, it is inevitable that the transfer efficiency during

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**Figure 3. A cartoon indicating the approach used by all three integration vector systems.**

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Replica plating is less than 100%. Furthermore, if the targeting event compromises fitness, vigorous growth of the non-transformed host clones may out compete the less fit transformant clones. We therefore sought recovery conditions in which cell division would be blocked and yet the antibiotic metabolising enzymes could accumulate in all transformants before exposure of the entire mix of transformants and untransformed neighbours to selection pressure.

Cells are unable to divide in the absence of a nitrogen source [64]. We therefore asked whether we could simply substitute the overnight incubation on solid medium with incubation in a liquid minimal medium that lacked a nitrogen source. The MSL medium that was developed by Richard Egel [65], is ideal for this goal.

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**Figure 4. The pINTL series of vectors for the expression of a gene of interest from the leu1 locus.** Cartoons depicting the structure of the indicated pINTL vectors.

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| Vector | Promoter | Tag | Cartoon of the tag and MCS |
|--------|----------|-----|--------------------------|
| pINTL81 | nmt81    | —   | Ndel,Sall,BamHl,Smal     |
| pINTL81PkC | nmt81 | C terminal, 3P | Ndel,Sall,KpnI,BglII,BamHl,Smal |
| pINTL41 | nmt41    | —   | Ndel,Sall,BamHl,Smal     |
| pINTL41PkN | nmt41 | N terminal, 3P | Ndel,Sall,KpnI,BglII,BamHl,Smal |
| pINTL41PkC | nmt41 | C terminal, 3P | Ndel,Sall,KpnI,BglII,BamHl,Smal |
| pINTL41HAN | nmt41 | N terminal, 3HA | Ndel,Sall,BamHl,Smal |
| pINTL41HMN | nmt41 | N terminal, HisMyc | Ndel,Sall,BamHl,Smal |
| pINTL41EGFPN | nmt41 | N terminal, EGFP | Ndel,Sall,BamHl,Smal |
| pINTL41EGFPC | nmt41 | C terminal, EGFP | Ndel,Sall,KpnI,BglII,BamHl,Smal |
| pINTL41GST   | nmt41 | N/C terminal, GST | Ndel,GST,BamHl,Smal |
| pINTL1      | nmt1    | —   | Ndel,Sall,BamHl,Smal     |
Figure 5. The pINTK series of vectors for the expression of a gene of interest from the \textit{lys1} locus. Cartoons depicting the structure of the indicated pINTK vectors.
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because it efficiently invokes a nitrogen starvation response. Amino acid supplements were omitted from this medium in our tests to limit provision of nitrogen from \textit{in vivo} amino acid catabolism.

We used the integration of a marker at the \textit{pku80} and \textit{leu1} loci to assess the impact of MSL-N recovery phase of differing durations upon the transformation efficiency. Cells were grown to mid log phase (4 \times 10^6 cells ml^{-1}) in rich YES medium before standard procedures were used to make the cells competent to receive DNA. The DNA fragments that were added to these competent cells were generated by PCR amplification of pFA6a antibiotic resistance deletion vector series templates with the same oligonucleotides being used with each template [40,58]. For each marker tested the transformed cell mix was split into 6. One portion was immediately spread onto non-selective YES plates at

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**Figure 6.** The pINTH series of vectors for the expression of a gene of interest from the \textit{hph.171k} locus on chromosome III. Cartoons depicting the structure of the indicated pINTH vectors.

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25°C, while the others were re-suspended in 1 ml of MSL-N and incubated with agitation at 25°C. The MSL-N transformation mixes were spread onto selective plates 2, 4, 8, 16 and 24 hours later. Cell counts confirmed that no cell division occurred during the 24 hours of incubation in MSL-N medium (data not shown). The lawn of cells on the YES plates that had received the

Figure 7. Integration at either the hph.171k or leu1 loci gave identical levels of protein expression. A) Cartoons showing the structure of the two nmt41 integrated cassettes from which catalytically inactive Fin1.KD fusion proteins (three “Pk” SV5 epitopes fused, in frame, to their amino termini) are expressed upon removal of thiamine. B) Cells were grown to early log phase in EMM2+15 μM thiamine at 25°C before being washed three times in thiamine free EMM2 medium and re-suspended in EMM2 at a density of 1.8 x 10⁵. Protein extracts were prepared from the mid-log phase cultures and processed for Western Blots after a further 15 hours culture at 25°C. Blots were cut in two; high molecular weight regions were probed with Fin1 antibodies while the loading control, While Cdc2 was detected on the lower molecular weight portion of the same blot. C) The same samples as shown in B probed with Cdc2 and mAb336 antibodies that recognised the Pk tags on the Fin1.KD3Pk fusion protein. D) A plot of the intensity ratios between the Fin1 and Cdc2 bands in each lane of the blots in B setting the ratio seen in wild type cells as 1 and that detected in fin1Δ control as 0.

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transformation mix immediately were replica plated 20 hours after the initial spreading of the transformation mix. There were no major differences in the number of transformants between any of the protocols when uracil prototrophy was used as the selectable marker to detect \( \text{ura}^+ \) integration (Table 1). In contrast, when antibiotic resistance formed the basis for the selection for the

Figure 8. The pREP\textsuperscript{N} series of vectors for the expression of a gene of interest from an ectopic plasmid. Cartoons depicting the structure of the indicated pREP\textsuperscript{N} vectors.
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| Vector       | Promoter | Tag     | Cartoon of the tag and MCS |
|--------------|----------|---------|-----------------------------|
| pRep8N       | nmt81    | —       | Ndel.SalI.HindII.XbaI.BamHI.Smal |
| pREP8N-PkN   | nmt81    | N terminal, 3Pk | Sacl Ncol Pk Pk Pk Pk Ndel.SalI.KpnI.BglII.XhoI.BamHI.Smal |
| pREP8N-PkC   | nmt81    | C terminal, 3Pk | Ndel.SalI.KpnI.BglII.XhoI.BamHI.Smal NcoI Pk Pk Pk |
| pRep4N       | nmt41    | —       | Ndel.SalI.HindII.XbaI.BamHI.Smal |
| pREP4N-PkN   | nmt41    | N terminal, 3Pk | Sacl Ncol Pk Pk Pk Pk Ndel.SalI.KpnI.BglII.XhoI.BamHI.Smal |
| pREP4N-PkC   | nmt41    | C terminal, 3Pk | Ndel.SalI.KpnI.BglII.XhoI.BamHI.Smal NcoI |
| pREP4N-EGFPN | nmt41    | N terminal, EGFP | NcoI EGFP Ndel.SalI.BamHI.Smal |
| pREP4N-EGFPC | nmt41    | C terminal, EGFP | Ndel.SalI.KpnI.BglII.XhoI.BamHI.Smal NcoI NcoI EGFP |
| pREP4N-HAN   | nmt41    | N terminal, 3HA | NcoI NcoI HA HA HA Ndel.SalI.BamHI.Smal |
| pRepN        | nmt1     | —       | Ndel.SalI.HindII.XbaI.BamHI.Smal |
| pREP1N-HMn   | nmt1     | N terminal, HisMyc | NcoI NcoI his(my) myc(my) Ndel.SalI.BamHI.Smal |
integration event between 5 to 8 fold more transformants were obtained in the samples that received a 24 hour MSL-N recovery period than when the aliquot had been replica plated aliquot (Table 1). PCR analysis revealed a similar rate of integrative transformation in either the replica plated or liquid recovery samples (data not shown).

![Cartoons depicting the structure of the indicated pREPK vectors.](doi:10.1371/journal.pone.0097683.g009)
Enhanced Efficiency of Integrative Targeting in pku70Δ or pku80Δ Backgrounds

Our attempts to target integration at a range of loci concur with the anecdotal experiences of the S. pombe community that the efficiency of targeting different loci varies widely. In many fungi removal of the Ku70 and Ku80 end recognition proteins blocks non-homologous end joining DNA repair pathway [49] to greatly enhance the frequency of gene targeting [50,51,52,53,54,55,56]. We therefore asked whether deletion of either molecule might enhance the 5% efficiency of integration at the S. pombe fin1+ locus.

Two types of DNA fragment were used for transformation: a large fragment excised from a plasmid in which the natMX6 marker was flanked by extensive regions of homology (1.2 kb 5' and 0.8 kb 3') to the fin1+ locus (Figure 2A, upper “fin1+ ORF”) and a short fragment with 80 bp regions of homology either side of the stop codon that generated a fin1+.3GFP fusion sequence by standard PCR amplification [40] from the pSM1023 template [66,67] (Figure 2A, lower “fin1+3GFP”). For the “fin1+ ORF” DNA fragment, a single sample of donor DNA was split into four. One quarter was transformed into a pku70::kanMX6 strain, another into an otherwise isogenic pku70+ strain and the remaining two aliquots into pku80::ura4+ and isogenic pku80+ hosts. As the selectable marker for the “fin1+3GFP” fragment was the same genetic/G418 resistance marker that had been used to delete pku70+ with kanMX6, this fin1+.3GFP fragment was only transformed into pku80::ura4+ and isogenic hosts. Diagnostic PCR analysis of transformants from each comparison revealed that the efficiency of gene targeting was elevated to between 75 and 80% (at least 16 fold increase) by the removal of either Pku70 or Pku80 (Figure 2B, C). Such a marked improvement in transformation efficiency upon removal of these end recognition factors prompted us to generate strains in which pku70+ and pku80+ have been replaced with the kanMX6, hphMX6 and natMX6 cassettes. These strains have been deposited in the Yeast Genome Resource Centre Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html, for YGRC strain numbers see Table 2).

Integration Vectors

Although the expression of molecules from multi-copy vectors can be highly informative, the highly variable stoichiometry of protein levels between neighbouring cells can make it difficult to derive concrete conclusions from a particular manipulation. In contrast, direct comparisons can be made between the consequences of expressing different mutant alleles when integrated in the same vector context into the same genomic location. We have therefore developed three different vector series that each direct integration of an expression cassette into distinct, defined locations in the fission yeast genome. The same principle is employed in the selection of the pINTH expression cassettes at uC, uD, uF and uG allele that encodes a catalytically inactive kinase, as a target site for vector integration. As the smallest chromosome, chromosome III, harbours the smallest proportion of the genome of the three chromosomes, inserting a marker on this chromosome would lend itself to easier manipulation in subsequent crosses to introduce an expression cassette into a particular background. We therefore scanned chromosome III using the dataset of Wilhelm et al. to find regions with low or no transcriptional activity [69]. Because no transcription was detected around position 171385 we integrated the Hygromycin B resistance cassette, hphMX6, at this site to generate an integration target locus that we refer to as the “hph.171k” locus (available from Yeast Genome Resource Centre Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html). YGRC strain number listed in Table 2). The growth rate and fitness of hph.171k cells was indistinguishable from wild type at all temperatures tested (20°C, 25°C, 30°C, 32°C, 36°C) in both rich YES and minimal EMM2 medium. We then generated the pINTH series of vectors shown in Figure 6B that can be used to target any integration to any hphMX6 sequence in the genome (Figure 6B). In our case we use it to target hph.171k.

To assess the level of expression obtained following integration of the pINTH expression cassettes at hph.171k we cloned a fin1 allele that encodes a catalytically inactive kinase, fin1.KD [62], into the pINTH1PKN vector. The NolI restriction enzyme digested fragment was transformed into a hph.171k host (Figure 7A). 135 of the 141 transformants obtained were hygromycin resistance negative and nourseothricin resistance positive (i.e. a targeting efficiency of 96% in this hph.171k pku70+ pku80+ host). After backcrossing, protein samples were prepared from mid-log phase cultures 15 hours after expression from the nmt41 promoter was de-repressed by the removal of thiamine and processed for western blotting. To compare the expression level of the pINTH vectors with that obtained with the pINTL series vectors, the same fin1.KD insert had been cloned into the pINTL41PKN vector before integration into the genome, backcrossing and the production of protein extracts from mid-log phase cultures 15 hours after thiamine removal. The levels of Fin1.KD protein attained following induction of expression from either the nmt1 targeted

Auxotrophic plNLT and plNTK Integration Vectors

In the INTL vectors the leu1+ gene has been disrupted by a cloning module (expression or expression + tag) and ura4+ (Figure 4). The entire cassette is flanked by NolI restriction sites. Once the desired sequences have been inserted, the Nol fragment is excised and transformed into an ura4Δ strain. In correct transformants the disruption of leu1+ by the vector sequences flips auxotrophy from leu+ to leu− and ura4+ to ura−. INTK vectors direct the disruption of lys1+ with a similar ura4+ expression/tagging module to switch auxotrophy from lys+ ura− to lys− ura+ (Figure 5). The use of ura4+ as a positive selection in both the INTL and INTK systems excluding subsequent use of ura4+ based multi-copy vectors is an advantage. Consequently, the ura4+ sequences within the INTK cassette have been flanked with loxP sites to facilitate marker excision upon expression of Cre recombinase (Figure 5).

Nourseothricin Resistance Based pINTH Integration Vectors

Modification of the amino acid requirements and amino acid content of the medium can significantly change flux through the TOR signalling pathway to impact upon diverse aspects of cell physiology [28]. We therefore generated an integrative vector system that can be used in prototrophs because it relies upon switching resistance to antibiotics rather than amino acid requirements. To achieve this goal we needed to select a site at which to integrate the recipient antibiotic marker that would later act as a target site for vector integration. As the smallest chromosome, chromosome III, harbours the smallest proportion of the genome of the three chromosomes, inserting a marker on this chromosome would lend itself to easier manipulation in subsequent crosses to introduce an expression cassette into a particular background. We therefore scanned chromosome III using the dataset of Wilhelm et al. to find regions with low or no transcriptional activity [69]. Because no transcription was detected around position 171385 we integrated the Hygromycin B resistance cassette, hphMX6, at this site to generate an integration target locus that we refer to as the “hph.171k” locus (available from Yeast Genome Resource Centre Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html). YGRC strain number listed in Table 2). The growth rate and fitness of hph.171k cells was indistinguishable from wild type at all temperatures tested (20°C, 25°C, 30°C, 32°C, 36°C) in both rich YES and minimal EMM2 medium. We then generated the pINTH series of vectors shown in Figure 6B that can be used to target any integration to any hphMX6 sequence in the genome (Figure 6B). In our case we use it to target hph.171k.

To assess the level of expression obtained following integration of the pINTH expression cassettes at hph.171k we cloned a fin1 allele that encodes a catalytically inactive kinase, fin1.KD [62], into the pINTH1PKN vector. The NolI restriction enzyme digested fragment was transformed into a hph.171k host (Figure 7A). 135 of the 141 transformants obtained were hygromycin resistance negative and nourseothricin resistance positive (i.e. a targeting efficiency of 96% in this hph.171k pku70+ pku80+ host). After backcrossing, protein samples were prepared from mid-log phase cultures 15 hours after expression from the nmt41 promoter was de-repressed by the removal of thiamine and processed for western blotting. To compare the expression level of the pINTH vectors with that obtained with the pINTL series vectors, the same fin1.KD insert had been cloned into the pINTL41PKN vector before integration into the genome, backcrossing and the production of protein extracts from mid-log phase cultures 15 hours after thiamine removal. The levels of Fin1.KD protein attained following induction of expression from either the nmt1 targeted
pINTL41PlN or the hph.17k targeted pINTH41PlN construct (Figure 7B, C) were indistinguishable from one another (3 fold higher than that of the native Fin1 kinase (Figure 7B)). As expected, the expression levels from the nmt81 based cassette was lower than from the nmt41 cassettes. We note that the 2 fold differential in protein levels between the two strength promoters (Figure 7D) is less than the 10 fold difference reported for the production of RNA levels from the nmt81 and nmt41 promoters on multi-copy vectors [70], however, Fin1 is subject to proteolytic control [62] making it impossible to draw solid conclusions about transcription rates when integrated into the *lew1* locus.

### pREP1N and pREPK Vectors

Multi-copy plasmids that can be selected for in prototrophs to drive the expression of tagged molecules remain popular. To generate a series of vectors we used *in vivo* gap repair in fission yeast [24,71] to switch the markers in pREP81 and pREP41/42 based vector backbones [68]. We have previously reported the construction of these donor vectors [16]. Of the four antibiotic resistance markers in use in *S. pombe*, only the *nmt1*, *Ura4* and *Kan* based cassette was used in the normal medium in which the nmt1 based promoters of the pREP series vectors can be de-repressed, making these the only markers that would be of utility for a pREP based series of vectors. The *LEU2* marker of pREP81 and pREP41 derived plasmids was removed by restriction digestion and the linear vector sequences were co-transformed with a DNA fragment in which the *natMX6* or the *kanMX6* cassette had been amplified with primers that had 80 bp of homology with either end of the opened vector sequences. Plasmids were re-isolated from two nat + or kan + transformants and the new vector sequenced. As reported previously for this approach [24,71], recombination had faithfully created the desired vectors in each case (Figure 8, 9). The transformation efficiency and stability of the pREP1N plasmid was indistinguishable from that of pREP1 (data not shown).

### Discussion

We describe a number of the tools that we have developed to assist our efforts that exploit the molecular genetics of *S. pombe* to understand the signal transduction pathways that control cell division. The tools and methods presented in this paper make a significant contribution to resolving the problem of locus-dependent gene targeting efficiency. Manipulation of all loci has now become routine with the enhancements of transformation efficiency after switching the recovery incubation to an overnight incubation in un-supplemented MSL and the removal of the NHEJ response by deleting either pka70 or pka80 (the choice of which deletion to use depends upon the genomic location of the gene of interest to be targeted). We have generated pkaX0:-*kanMX6*, pkaX0:*hphMX6*, pkaX0:*natMX6* strains for greatest flexibility in designing a particular knockout strategy [available from the Yeast Genome Resource Centre Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html)]. We note that *ura4* and *LEU2* deleted alleles have been generated in other studies [72,73].

While removal of the NHEJ pathway radically enhances the frequency of gene targeting in our work on cell cycle control, our experience with genes in the TOR signalling pathway has been different as targeting can be less efficient in pka70A and pka80A strains than in wild type strains (data not shown). Why this should be is unclear, however we suggest that pka70* and pka80* deletions be used as host strains for manipulations of genes in processes other than TOR signalling, but reverting to targeting in a wild type prototrophic strain should targeting efficiencies prove to be poor.

We found that the efficiency of integration was radically enhanced by altering the nature of the recovery period that is used to enable the expression of antibiotic resistance markers before they are challenged with selective conditions. Switching a from recovery phase on solid medium to a liquid recovery phase in nitrogen free medium increased transformation efficiencies 5-8 fold with antibiotic resistance cassettes. Although the greatest enhancement of transformation efficiencies arose at the 24 hour time point, an overnight incubation is normally sufficient and avoids delays in strain construction. While the MSL-N recovery period was a great benefit when the integrated expression cassette directed the expression of antibiotic resistance markers to counteract the otherwise lethal impact of antibiotics, it had only a modest impact when the selection relied upon *ura4* complementation of *ura4.d18* in media lacking uracil. We assume that this is due to the inherent differences in the nature of the selection pressure in the two cases. Expression of antibiotic resistance molecules is required to prevent an apparently immediate death from a lethal assault by the antibiotic, whereas the ornithine decarboxylase is required to permit the generation of uracil. Cells will simply remain in a stationary phase until ornithine decarboxylase levels reach the critical threshold to allow them to resume growth and division.

The generation of three series of integration vectors that allow the expression of wild type, mutant, tagged or un-tagged native molecules from three different loci greatly facilitates the analysis of the impact of mutations on individual molecules or compound interactions of mutant molecules in a protein complex. The pINTXXX.A vectors can accept the entire promoter–gene–terminator cassette as a PsI-SacI restriction fragment from any existing pREP based vector [74]. Furthermore, the cloning approaches can be adapted to express full length non-coding RNAs from these sites of integration [75]. While we describe the full vector series here, we have used some members of each series in a number of studies that validate the application of these vectors in molecular cell biology in fission yeast [62,74,76,77,78,79,80,81,82,83,84].

### Supporting Information

Figure S1 DNA sequences of the pINTL series. (DOCX)

Figure S2 DNA sequence of pINTK. (DOCX)

Figure S3 DNA sequences of the pINTH series. (DOCX)

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### Author Contributions

Conceived and designed the experiments: DF AG AK JP VS IMH. Performed the experiments: DF AG AK AC. Analyzed the data: DF AG AK VS IMH. Contributed reagents/materials/analysis tools: DF AG AK AC AJB JP AP VAT EB BH. Contributed to the writing of the manuscript: IMH VS AG AK.
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