

FRAGMENT SWITCH: A REVERSE GENETIC APPROACH

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application is related to and claims priority to U.S. provisional patent application Serial No. 61/329,888 filed on 30 April 2010.

SEQUENCE SUBMISSION

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577-202PCT_ST25.txt, was created on 11 March 2011 and is 7.32 kb in size. The information in the electronic format of the Sequence Listing is part of the present application and is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0003] The present invention relates to the field of reverse genetics. More particularly, the present invention relates to a novel reverse genetic approach termed "fragment switch" which is used to generate an allelic series in genes of interest which are useful for functional analysis.

[0004] The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the Bibliography.

[0005] Genetic analysis using mutant strains have played important roles in the identification and functional analysis of genes. The phenotype-based forward genetic screens have contributed a lot to discovering genes involved in biosynthesis, cell cycle, and signaling pathways etc. In order to dissect multiple functions of some genes and the underlying molecular mechanisms, an allelic series becomes desirable. With the genomes sequenced, reverse genetic screens have become prevalent tools for generating mutant alleles, especially for genes that have not been identified by forward genetic screens.

[0006] There are several reverse genetic approaches for fission yeast, such as plasmid shuffling (Liang and Frosburg, 2001), degron system (Kanemaki et al., 2003), chromosomal integration and marker switch (MacIver et al., 2003). All these techniques have their own limitations. Plasmid shuffling is time consuming. Degron system creates

only one loss of function temperature sensitive allele, and does not work for all genes because it requires an additional peptide to be tagged. Both chromosomal integration and marker switch use polymerase chain reaction (PCR) to amplify a long fragment that consists of a flanking sequence, a selective marker gene and the target gene (Fig. 1a). The long selective marker gene not only leads to less PCR product, but also has high chance to get unexpected mutations by mutagenic PCR, leading to inefficient swapping mutation into the target gene.

[0007] Therefore, a simpler and more efficient approach is desirable for reverse genetics.

SUMMARY OF THE INVENTION

[0008] The present invention relates to the field of reverse genetics. More particularly, the present invention relates to a novel reverse genetic approach termed "fragment switch" which is used to generate an allelic series in genes of interest which are useful for functional analysis.

[0009] In one aspect, the present invention provides a method for generating an allelic series in a gene of interest. In one embodiment, a method for generating a mutant allele of a gene of interest comprises integrating a first nucleic acid fragment adjacent to a gene of interest in a first nucleic acid in a host organism's DNA, wherein the first nucleic acid fragment comprises (i) a gene encoding a first selectable marker having a carboxy terminus truncation or an amino terminus truncation and (ii) a gene encoding a second selectable marker. The method also comprises preparing a second nucleic acid fragment comprising (i) the gene of interest having one or more mutations and (ii) a fragment of the first selectable marker gene, wherein the fragment of the first selectable marker gene encodes the carboxy terminus or amino terminus of the first selectable marker. The method further comprises recombining the second nucleic acid fragment with the first nucleic acid under pressure of selection for the first selectable marker to produce a second nucleic acid comprising (i) the gene of interest having the one or more mutations, (ii) a gene encoding the first selectable marker and (iii) a gene encoding the second selectable marker.

[0010] In one embodiment, the nucleic acid fragment encoding the first selectable marker having a carboxy terminus truncation or an amino terminus truncation is integrated next to the gene of interest. In one embodiment, the truncation is a carboxy terminus

truncation and the 3' end of the gene encoding the first selectable marker is positioned on the 3' side of the gene of interest. In another embodiment, the truncation is a carboxy terminus truncation and the 3' end of the gene encoding the first selectable marker is positioned on the 5' side of the gene of interest. In an additional embodiment, the truncation is an amino terminus truncation and the 5' end of the gene encoding the first selectable marker is positioned on the 3' side of the gene of interest. In a further embodiment, the truncation is an amino terminus truncation and the 5' end of the gene encoding the first selectable marker is positioned on the 5' side of the gene of interest.

[0011] In one embodiment, the fragment of the first selectable marker gene is located next to the gene of interest having the one or more mutations. In another embodiment, the carboxy terminus of the first selectable marker in the second fragment complements the first selectable marker having the carboxy terminus truncation or the amino terminus of the first selectable marker in the second fragment complements the first selectable marker having the amino terminus truncation. In one embodiment, the fragment of the first selectable marker gene encodes the carboxy terminus of the first selectable marker and the 3' end of the fragment of the first selectable marker gene is positioned on the 3' side of the gene of interest having the one or more mutations. In another embodiment, the fragment of the first selectable marker gene encodes the carboxy terminus of the first selectable marker and the 3' end of the fragment of the first selectable marker gene is positioned on the 5' side of the gene of interest having the one or more mutations. In an additional embodiment, the fragment of the first selectable marker gene encodes the amino terminus of the first selectable marker and the 5' end of the fragment of the first selectable marker gene is positioned on the 3' side of the gene of interest having the one or more mutations. In a further embodiment, the fragment of the first selectable marker gene encodes the amino terminus of the first selectable marker and the 5' end of the fragment of the first selectable marker gene is positioned on the 5' side of the gene of interest having the one or more mutations. In one embodiment, the second fragment can be produced by mutagenic PCR. In a further embodiment, the recombination is homologous recombination in a host organism.

[0012] In a second aspect, the present invention provides an isolated nucleic acid that comprises a gene encoding a first selectable marker having a carboxy terminus truncation or an amino terminus truncation and a gene encoding a second selectable marker. In one

embodiment, at least the portion of the isolated nucleic acid containing the first selectable marker gene and the second selectable marker gene is capable of integrating into a host organism's DNA. In another embodiment, the second marker gene is positioned on the 5' or 3' side of the first marker gene depending on which end of the first marker gene integrates next to the gene of interest.

[0013] In a third aspect, the present invention provides a nucleic acid that comprises a gene of interest, a gene encoding a first selectable marker having a carboxy terminus truncation or an amino terminus truncation and a gene encoding a second selectable marker. In one embodiment, the gene encoding the first selectable marker having a carboxy terminus truncation is integrated next to the gene of interest as described herein. In another embodiment, the gene encoding the first selectable marker having an amino terminus truncation is integrated next to the gene of interest as described herein.

[0014] In a fourth aspect, the present invention provides an isolated nucleic acid that comprises a gene of interest having one or more mutations and a fragment of the first selectable marker gene which encodes the carboxy terminus or amino terminus of the first selectable marker. In one embodiment, the fragment of the first selectable marker gene is located next to the gene of interest having the one or more mutations as described herein. In another embodiment, at least the portion of the isolated nucleic acid containing the gene of interest having one or more mutations and the fragment of the first selectable marker gene is capable of integrating into a host organism's DNA.

[0015] In a fifth aspect, the present invention provides a nucleic acid that comprises a gene of interest having one or more mutations, a gene encoding a first selectable marker and a gene encoding a second selectable marker.

BRIEF DESCRIPTION OF THE FIGURES

[0016] Figures 1a and 1b show marker switch and design of fragment switch. Fig. 1a: Marker switch. Selection marker 1 ($sm1^+$) is inserted adjacent to gene of interest (goi^+) through homologous recombination. Mutations (asterisk) generated by mutagenic PCR are delivered into chromosome together with the selection for selection marker 2 ($sm2^+$), which replaces $sm1^+$. Fig. 1b: A partial fragment ($sm1^{Δc}$) of $sm1^+$ with a carboxyl terminus truncation is inserted adjacent to goi^+ together with the selection for $sm2^+$. Mutations are

delivered into chromosome under the selection for *smI*⁺, which is complemented by the recombination between *smI*^{Δc} and *smI*^c, a fragment of the carboxyl terminus of *smI*⁺.

[0017] Figures 2a-2c show the two steps of fragment switch. Fig. 2a: Step one: plasmid-based strategy is used to insert *his5*^{Δc} adjacent to *goi*⁺. One upstream and one downstream fragments of the insertion locus, which have an overlap about 25 nucleotides and a unique restriction site (double arrows), are amplified, mixed, and then amplified again to generate a fusion fragment. This fusion fragment is cloned into plasmid *pH5ΔcU4*⁺. The resulting plasmid is restricted (scissor symbol) and transformed into a strain with the endogenous *his5*⁺ and *ura4*⁺ deleted, generating *goi*⁺-*his5*^{Δc}. Fig. 2b: Step two: mutations are delivered to *goi*⁺ together with the complementation of *his5*⁺. The whole *goi*⁺ is cloned into plasmid *pH5c*. Mutagenic PCR is used to produce the fusion fragment *goi*^m-*his5*^c, which is subsequently transformed and recombined with *goi*⁺-*his5*^{Δc}. Fig. 2c: Domain-specific mutagenesis: mutagenic PCR is applied to a specific region and high fidelity PCR to the downstream part, and a second round of high fidelity PCR again to generate a fusion fragment in which most mutations are in the this specific region.

[0018] Figure 3 shows mutants generated with fragment switch. Seven genes were picked to test the fragment switch approach. All mutants except *ppk37-24* were temperature sensitive in rich medium YES (die at 36° C but survive at 24° C). Mutants were cultured in YES over night at 24° C, and then shifted to 36° C for 4 hours. Cells were fixed with formaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI) (DNA) and aniline blue (septum). The *his5*^{Δc} was inserted in the 3'-UTR region of the genes except *myo2*, in which it was inserted in both 5'- and 3'-UTR separately in two strains.

DETAILED DESCRIPTION OF THE INVENTION

[0019] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention belongs.

[0020] The present invention relates to the field of reverse genetics. More particularly, the present invention relates to a novel reverse genetic approach termed "fragment switch"

which is used to generate an allelic series in genes of interest which are useful for functional analysis.

[0021] Mutagenesis by a reverse genetic approach requires optimal mutation frequency, efficient targeting, and selection pressure. Those could be fulfilled by mutagenic PCR (Vallette et al., 1989), homologous recombination and an efficient selective marker. As shown in Fig. 1a, marker switch approach consists of two steps: insertion of a first selectable marker ($sm1^+$) next to a gene of interest (goi^+) and incorporation of mutations into goi^+ together with the replacement of $sm1^+$ by a second selectable marker $sm2^+$. Actually, chromosome integration only uses the first step to deliver mutation into the gene of interest, but marker switch has an advantage because it indirectly targets mutation incorporation into goi^+ by excluding random integrations in other loci.

[0022] To simplify marker switch, it was combined with a targeted integration approach described previously (Keeney and Boeke, 1994). Two simple steps were designed to fulfill this approach (Fig. 1b). First, a fragment ($sm1^{Δc}$) of $sm1^+$ with a carboxyl terminus truncation is integrated adjacent to goi^+ together with another selection marker ($sm2^+$). Second, mutagenic PCR is used to produce a fusion fragment between goi and the carboxy terminus of $sm1^+$, goi^m-sm1^c , which harbors a mutation in goi and complements $sm1^{Δc}$. After this fusion fragment is transformed, mutations are delivered into goi through homologous recombination precisely between this fusion fragment and $goi^+-sm1^{Δc}$ under the pressure of selection for $sm1^+$. This eliminates the need to further confirm the swapping as done by marker switch. Since only a partial fragment of the selective marker instead of a full length is used for integration by homologous recombination, this approach is called "fragment switch" herein.

[0023] In one aspect, the present invention provides a method for generating an allelic series in a gene of interest. In one embodiment, a method for generating a mutant allele of a gene of interest comprises integrating a first nucleic acid fragment adjacent to a gene of interest in a first nucleic acid in a host organism's DNA, wherein the first nucleic acid fragment comprises (i) a gene encoding a first selectable marker having a carboxy terminus truncation or an amino terminus truncation and (ii) a gene encoding a second selectable marker. The method also comprises preparing a second nucleic acid fragment comprising (i) the gene of interest having one or more mutations and (ii) a fragment of the first

selectable marker gene, wherein the fragment of the first selectable marker gene encodes the carboxy terminus or amino terminus of the first selectable marker. The method further comprises recombining the second nucleic acid fragment with the first nucleic acid under pressure of selection for the first selectable marker to produce a second nucleic acid comprising (i) the gene of interest having the one or more mutations, (ii) a gene encoding the first selectable marker and (iii) a gene encoding the second selectable marker.

[0024] In one embodiment, the nucleic acid fragment encoding the first selectable marker having a carboxy terminus truncation or an amino terminus truncation is integrated next to the gene of interest. In one embodiment, the truncation is a carboxy terminus truncation and the 3' end of the gene encoding the first selectable marker is positioned on the 3' side of the gene of interest. In another embodiment, the truncation is a carboxy terminus truncation and the 3' end of the gene encoding the first selectable marker is positioned on the 5' side of the gene of interest. In an additional embodiment, the truncation is an amino terminus truncation and the 5' end of the gene encoding the first selectable marker is positioned on the 3' side of the gene of interest. In a further embodiment, the truncation is an amino terminus truncation and the 5' end of the gene encoding the first selectable marker is positioned on the 5' side of the gene of interest.

[0025] In one embodiment, the fragment of the first selectable marker gene is located next to the gene of interest having the one or more mutations. In another embodiment, the carboxy terminus of the first selectable marker in the second fragment complements the first selectable marker having the carboxy terminus truncation or the amino terminus of the first selectable marker in the second fragment complements the first selectable marker having the amino terminus truncation. In one embodiment, the fragment of the first selectable marker gene encodes the carboxy terminus of the first selectable marker and the 3' end of the fragment of the first selectable marker gene is positioned on the 3' side of the gene of interest having the one or more mutations. In another embodiment, the fragment of the first selectable marker gene encodes the carboxy terminus of the first selectable marker and the 3' end of the fragment of the first selectable marker gene is positioned on the 5' side of the gene of interest having the one or more mutations. In an additional embodiment, the fragment of the first selectable marker gene encodes the amino terminus of the first selectable marker and the 5' end of the fragment of the first selectable marker gene is positioned on the 3' side of the gene of interest having the one or more mutations.

In a further embodiment, the fragment of the first selectable marker gene encodes the amino terminus of the first selectable marker and the 5' end of the fragment of the first selectable marker gene is positioned on the 5' side of the gene of interest having the one or more mutations. In one embodiment, the second fragment can be produced by mutagenic PCR. In a further embodiment, the recombination is homologous recombination in a host organism.

[0026] In addition to mutagenesis, fragment switch could be used for other reverse genetic manipulation such as precise carboxyl terminal tagging and even deletion. For precise carboxy terminal tagging, this method comprises preparing the second nucleic acid fragment comprising (i) the gene of interest with the stop codon deleted, (ii) a tagging fragment (such as *gfp*) fused with the gene of interest in frame, (iii) a fragment of the first selectable marker gene which encodes the carboxy terminus of the first selectable marker. For precise deletion, this method comprises preparing the second nucleic acid fragment comprising (i) a fragment upstream a gene of interest, (ii) a fragment of the first selectable marker gene which encodes the carboxy terminus of the first selectable marker. The method of the present invention can be used in any host organism in which homologous recombination can be performed. In addition to yeast that is illustrated herein (*Schizosaccharomyces pombe*), other organisms include, but are not limited to the bacterium *Escherichia coli*, the yeast *Saccharomyces cerevisiae*.

[0027] The gene of interest may be any gene for which it is desired to create an allelic series that is useful in an analysis of gene function by a reverse genetic approach. In the fact, this method is applicable to any gene for mutagenesis as long as the phenotype of the mutants is observable. Examples of genes include, but are not limited to three classes of genes in fission yeast: (i) genes encoding kinases (Bimbo et al., 2005), such as *ppk37*, *ark1*, *cdc2*, *cdc7*, *cdk9*, *hsk1*, *orb6*, *plb1*, *ran1*, *shk1*, *sid1*, *sid2*, *ppk19*, and genes encoding phosphatase, including *cdc25*, *fcpl*. (ii) genes encoding proteins essential for the functional cellular cytoskeleton, including *act1*, *arp3*, *cdc12*, *rng2*, *myo2*, *rng3*, *adfl*, *nda3*, *nda2*, *alp1*, *alp21*, *alp6*, *alp4*, *pcp1*. (iii) genes involving in signaling cascade, such as genes for septum initiating network, *plb1*, *byr4*, *cdc16*, *spg1*, *cdc7*, *cdc11*, *sid4*, *sid1*, *sid2*.

[0028] The mutation may be any mutation that leads to a loss of function or a gain of function. Thus, the mutation may be a nonsense mutation, a frameshift mutation, an

insertion mutation, a deletion mutation or a missense mutation, each of which would lead to a loss of function or a gain of function.

[0029] Any selectable marker gene can be used in the present invention as long as it is compatible with and expressed in the host organism. Examples of selective markers in fission yeast include, but are not limited to, *his5+* and *ura4*. Examples of selectable markers in budding yeast include, but are not limited to, *HIS3+* and *URA3*. Examples of selectable markers in *Escherichia coli* include, but are not limited to, *bla*, *hyrR*, and *kanR*. In one embodiment, the gene encoding the selectable marker includes the native promoter. In another embodiment, the gene encoding the selectable marker includes a heterologous promoter. Any heterologous promoter may be used as long as it functions in the host organism.

[0030] In a second aspect, the present invention provides an isolated nucleic acid that comprises a gene encoding a first selectable marker having a carboxy terminus truncation or an amino terminus truncation and a gene encoding a second selectable marker. In one embodiment, at least the portion of the isolated nucleic acid containing the first selectable marker gene and the second selectable marker gene is capable of integrating into a host organism's DNA. In another embodiment, the second marker gene is positioned on the 5' or 3' side of the first marker gene depending on which end of the first marker gene integrates next to the gene of interest. The selectable markers may be a selectable marker as described herein.

[0031] In a third aspect, the present invention provides a nucleic acid that comprises a gene of interest, a gene encoding a first selectable marker having a carboxy terminus truncation or an amino terminus truncation and a gene encoding a second selectable marker. In one embodiment, the gene encoding the first selectable marker having a carboxy terminus truncation is integrated next to the gene of interest as described herein. In another embodiment, the gene encoding the first selectable marker having an amino terminus truncation is integrated next to the gene of interest as described herein. The gene of interest may be a gene as described herein. The selectable markers may be a selectable marker as described herein.

[0032] In a fourth aspect, the present invention provides an isolated nucleic acid that comprises a gene of interest having one or more mutations and a fragment of the first selectable marker gene which encodes the carboxy terminus or amino terminus of the first

selectable marker. In one embodiment, the fragment of the first selectable marker gene is located next to the gene of interest having the one or more mutations as described herein. In another embodiment, at least the portion of the isolated nucleic acid containing the gene of interest having one or more mutations and the fragment of the first selectable marker gene is capable of integrating into a host organism's DNA. The gene of interest may be a gene as described herein. The selectable markers may be a selectable marker as described herein. The mutation may be a mutation as described herein.

[0033] In a fifth aspect, the present invention provides a nucleic acid that comprises a gene of interest having one or more mutations, a gene encoding a first selectable marker and a gene encoding a second selectable marker. The gene of interest may be a gene as described herein. The selectable markers may be a selectable marker as described herein. The mutation may be a mutation as described herein.

[0034] The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis *et al.*, 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Ausubel *et al.*, 1992), *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Russell, 1984, *Molecular biology of plants: a laboratory course manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Methods In Enzymology*, Vols. 154 and 155 (Wu *et al.* eds.), *Immunochemical*

Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Fire et al., *RNA Interference Technology: From Basic Science to Drug Development*, Cambridge University Press, Cambridge, 2005; Schepers, *RNA Interference in Practice*, Wiley-VCH, 2005; Engelke, *RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology*, DNA Press, 2003; Gott, *RNA Interference, Editing, and Modification: Methods and Protocols (Methods in Molecular Biology)*, Human Press, Totowa, NJ, 2004; Sohail, *Gene Silencing by RNA Interference: Technology and Application*, CRC, 2004.

EXAMPLES

[0035] The present invention is described by reference to the following Examples, which is offered by way of illustration and is not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Preparation of Plasmids for Fragment Switch in Yeast

[0036] Two plasmids, *pH5ΔcU4+* (Fig. 2a) and *pH5c* (Fig. 2b), were constructed for the integration of *sm1^{Δc}* next to *goi⁺* and generation of the fusion fragment *goi^m-sm1^c*, respectively. Plasmid pN1 was constructed by PCR-based amplifying and circulizing a fragment using pUC18 (Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed.) as the template and two overlapping primers (tcgactagtctcccggtatcctcaggcctcgtgatacgctattt (SEQ ID NO:1) and tcccgg gaggactagtgcacgttatcggcgagcggtatcagctcac (SEQ ID NO:2)). The vector *pH5ΔcU4+* was constructed by sequentially adding the functional selective maker *ura4+* and the nonfunctional selective marker *his5^{Δc}*, and a linker with multiple cloning sites (gtcgacagctgcggaattctgctagcggat ccagatct (SEQ ID NO:3)) into pN1. The plasmid *pH5c* was constructed by sequentially adding the carboxy terminus of *his5+* selective marker and the same linker in *pH5ΔcU4+*. The selection marker *his5⁺* was chosen as *sm1⁺* due to its effectiveness obtained during transformation (Tang et al., 2006) and its short coding sequence (651 base-pairs; GenBank Accession No. NM_001021840).

In *his5^{Δc}*, fifty base-pairs of the carboxyl terminus were removed, which made it nonfunctional. To insert *his5^{Δc}* adjacent to *goi⁺*, we used a plasmid-based integration strategy (Wang et al., 2004). As shown in Fig. 2a, PCR was used to generate one upstream and one downstream fragment of the insert locus separately. These two fragments overlapped 20~30 base-pairs in their outward ends (the double head-to-head arrows), which carry a unique endonuclease restriction site. These two fragments were mixed and used as the templates for the next round of fusion PCR. The fusion PCR product was cloned into the vector *pH5ΔcU4+*. The resulting plasmid was restricted, generating two ends that fully matched with the sequences on the chromosome. The linearized plasmid was transformed into the host organism (*Schizosaccharomyces pombe*) using the method as described (Okazaki, K. et al., 1990) and forced to undergo homologous recombination by the selection for *ura4⁺*, resulting in the integration of *his5^{Δc}* next to *goi⁺*. To introduce mutation into *goi⁺*, it was cloned into the vector *pH5c* to generate a fusion (*goi⁺-his5^c*) between *goi⁺* and a 400-base-pair fragment containing a 3'-UTR and the carboxyl terminus part of *his5⁺*. The fusion fragment *goi⁺-his5^c* was used as the template to amplify *goi^m-his5^c* by mutagenic PCR using one primer (MOH3724his5c25u: gacttgctcgggacggcctatgc; SEQ ID NO:4) specific to the 5' end of *goi⁺* and one specific to *his5c*. After *goi^m-his5^c* was transformed and recombined with *goi⁺-his5^{Δc}*, mutations were delivered to *goi* upon the selection for *his5⁺*.

EXAMPLE 2

Preparation of Mutant Alleles of Genes of Interest

[0037] To test fragment switch, we performed proof-of-principle experiments to isolate mutant alleles for several genes of interest. We picked five genes essential for cytokinesis (Balasubramanian et al., 2004), *cdc12*, *cdc15*, *rng2*, *plp1*, *myo2*, one gene essential for spindle formation and function (Hagan and Yanagida, 1995), *sad1*, and one gene encoding a kinase with unknown function (Bimbó et al., 2005), *ppk37* (Fig. 3). All the strains were grown in standard medium (Moreno et al., 1991) and transformation of *Schizosaccharomyces pombe* was done as described (Okazaki, K. et al., 1990). Details of genes of interest and the primers used are listed in Table 1.

TABLE 1

Genes of Interest and Primers

cdc12: SPAC1F5.04c, encoding Cdc12p, which is required for the assembly of the actin-myosin ring for cytokinesis. Cdc12p nucleates actin filaments that grow rapidly from their barbed ends in the presence of profilin.

Primers*	#1	GGCGGCGATATCGAAAGAGCTGGCTCGTTTGAC (SEQ ID NO:5)
	#2	CCGAGAGACAGCTGCAACAGCACAATGTATTTG (SEQ ID NO:6)
	#3	GCTGTTGCAGCTGTCTCTCGGAACCTTA (SEQ ID NO:7)
	#4	GCTATACGATATCCGGATCCAACTAACTAACTTCCAAA (SEQ ID NO:8)
	#5	GCTCCGAGTTAATTCGTGGT (SEQ ID NO:9)

cdc15: SPAC20G8.05c, encoding Cdc15p, which is required for assembly and stabilization of the actin-myosin ring for cytokinesis.

Primers*	#1	GGCGGGCAGCTGATGACTTAACGCGAAACGA (SEQ ID NO:10)
	#2	ATCTGTGACCCGGGTTTGCACCTCTTCAAACAT (SEQ ID NO:11)
	#3	TGCAAACCCGGGTCACAGATATGGGTCTAT (SEQ ID NO:12)
	#4	GCTATACGATATCCGGATCCACGCGAAAAAGAATGCAAG (SEQ ID NO:13)
	#5	CAAACATGTGGGCCTATGCA (SEQ ID NO:14)

rng2: SPAC4F8.13c, encoding Rng2p, which is required for assembly and contraction of the actin-myosin ring for cytokinesis.

Primers*	#1	GGAGGCCTCGAGTCTCTTTAACTACCCAATG (SEQ ID NO:15)
	#2	TATTACGGATATCCAAAGGTTAATTTGAACAT (SEQ ID NO:16)
	#3	AACCTTTGGATATCCGTAATAAGTCGAG (SEQ ID NO:17)
	#4	CTCGGAGCAGCTGAACAAAGTGAAACGTCTC (SEQ ID NO:18)
	#5	GAGGGCACTTCCTCTGTAAAGATCCGTCATGC (SEQ ID NO:19)

plol: SPAC23C11.16, encoding Plolp, which is required to form a bipolar spindle, the actin ring and septum, and for septal material deposition.

Primers*	#1	GGCGGAGATATCCATATTTACATCCTCATTTT (SEQ ID NO:20)
	#2	TCCTCATCAGCTGTGATTCTTAATATGCAGC (SEQ ID NO:21)
	#3	AAGAATCACAGCTGATGAGGAGGTTGTC (SEQ ID NO:22)
	#4	GTATGCTATACGATGGATCCAGCATAGTAACTTAACGCC (SEQ ID NO:23)
	#5	GGAGGAGATATCGTTGTCCCTTTCTTTGC (SEQ ID NO:24)

sad1: SPBC12D12.01, encoding Sad1p, which is a component of the spindle pole body and is involved in linking telomeres to the spindle pole body during meiotic prophase.

Primers*	#1	GGCGGAGATATCTGCTTCCTCGAGCATCA (SEQ ID NO:25)
	#2	TAATTGTCCCGGGCAAAACACTAATATTCGC (SEQ ID NO:26)
	#3	TGTTTTGCCCGGGACAATTAGGAATTTCCC (SEQ ID NO:27)
	#4	GCTATACGATATCCGGATCCAAACATTCCAGGCTAGATT (SEQ ID NO:28)
	#5	GGCATTCAATTACAGAATCC (SEQ ID NO:29)

ppk37: SPCC70.05c, encoding a kinase, which is essential for viability and which function is not known.

Primers*	#1	GGAGGAGTCGACCTAAGCTATTTATTTGG (SEQ ID NO:30)
	#2	AAACAATCAGCTGCTGTGGAATCAAAGAGT (SEQ ID NO:31)
	#3	TCCACAGCAGCTGATTGTTTATACTAATATC (SEQ ID NO:32)
	#4	GGAGGAAGATCTCACAAGTGATTGTGTGG (SEQ ID NO:33)
	#5	GGAGGACAGCTGTATGAAAAGCAGTGTTTG (SEQ ID NO:34)

myo2: SPCC645.05c, encoding Myo2p, which is involved in generating force for actin-myosin ring constriction, and also binds to *cdc4* and *rlc1*.

Primers	#1	GGAGGACTCGAGCTGTACATAATATTTCCCGC (SEQ ID NO:35)
for amino-	#2	TATCAACCAGCTGTGATCGCATTCATAGCCG (SEQ ID NO:36)
terminus*	#3	TGCGATCACAGCTGGTTGATACTCGAAAGG (SEQ ID NO:37)
	#4	GGAGGAGATATCATTTAACCTTCATGCGGCG (SEQ ID NO:38)
	#5	GCGCACCTTTAATAGACCCCT (SEQ ID NO:39)
Primers	#1	CCGCTCGAGTCGGCAAGTTTATGCAACTTG (SEQ ID NO:40)
for	#2	CCTACGTTTCATTTGATGCTGTCATGGATATCTCATTGC (SEQ ID NO:41)
carboxy	#3	GATATCCATGACAGCATCAAATGAACGTAGGATTAGGGG (SEQ ID NO:42)
terminus*	#4	GGAGGACAGCTGGTTATGGTATTCAGCATG (SEQ ID NO:43)
	#5	ATTCTGCTAAACACCT (SEQ ID NO:44)

* Primer pair of #1 and #2, pair of #3 and #4, were used to amplify the outwards and inwards fragment of the gene of interest from the insertion site of *his5^{Δc}-ura4⁺*. Then the fusion fragment of these two fragments was amplified using primer #1 and #4 and then cloned into *pH5ΔcU4⁺*. To construct fusion *goi⁺-his5^c*, primer #5 and #4 were used to amplify, which was then cloned into *pH5c*.

[0038] We obtained three *rng2* mutants from less than one hundred *his5⁺* transformants, one displayed temperature sensitive with multiple nuclei at 36° C. We did small-scale screenings for *cdc12*, *sad1*, and *ppk37* using only two plates (150 mm diameter), and collected 11, 28, 30 mutants respectively. Two of each are shown in Fig. 3. Both *cdc12-3* and *cdc12-6* died at 36° C; *cdc12-3* showed completely loss of septum formation, but *cdc12-6* showed partial formation and disorganized septum. Both *sad1* mutants showed unequal segregation of chromosomes, but *sad1-54* showed multiple DNA foci in most of the cells at 36° C. Most of the *ppk37* mutants showed slow growth in rich medium, arrested in different cell cycle stage like *ppk37-17* at 36° C, and lysed on agar medium (data not shown). Therefore, *ppk37* could be involved in membrane structure assembly.

Six mutants like *ppk37-24* showed double length as wild type and defects in septum formation. Most of *plo1* mutants were elongated with multiple nuclei and defective septum formation except that *plo1-83* only showed multiple nuclei and other four only showed defective in septum positioning (data not shown). Because *myo2* has a long coding sequence (4581 base-pairs), *his5^{Δc}* was either inserted in the 3'UTR for mutagenesis in the carboxyl terminus of *myo2*, or upstream of promoter for mutagenesis in the amino terminus. Most of the *myo2* mutants showed multiple nuclei and defective septum formation as in *myo2-c31* and *myo2-n1*.

[0039] The method of the present invention as illustrated in these Examples is simple, consisting of only two steps. In the first step, the integration of *his5^{Δc}* next to *goi⁺* is efficient because the insertion does not disrupt the function of *goi⁺*. In the second step, because only a short carboxyl terminus fragment *his5^c* is used to complement *his5^{Δc}*, homologous recombination has to undergo precisely between *goi^m-his5^c* and *goi⁺-his5^{Δc}*, avoiding integration at any other loci. Compared to marker switch, fragment switch provides a much easier way to control the most important part: mutagenic PCR. This is because fragment switch only need about 200 base-pairs of the carboxyl terminus of the selection marker, which is hardly incorporated with mutations by PCR. We tested fragment switch on several genes, even on the carboxyl terminal and amino terminal regions of *myo2*. Also, mutations could be primarily introduced into a small domain using fusion PCR (Fig. 2c), which will be useful for domain functional analysis. In addition to mutagenesis, fragment switch could be used for other reverse genetic manipulation such as precise carboxyl terminal tagging and even deletion. It could be adapted to other model organisms in which homologous recombination can be performed.

[0040] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value

falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. For example, if the range 10-15 is disclosed, then 11, 12, 13, and 14 are also disclosed. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0041] It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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WHAT IS CLAIMED IS:

1. A method of generating an allelic mutant in a gene of interest comprising:
 - (a) integrating a first nucleic acid fragment adjacent to a gene of interest in a first nucleic acid, wherein the first nucleic acid fragment comprises (i) a gene encoding a first selectable marker having a carboxy terminus truncation or an amino terminus truncation and (ii) a gene encoding a second selectable marker;
 - (b) preparing a second nucleic acid fragment comprising (i) the gene of interest having one or more mutations and (ii) a fragment of the first selectable marker gene, wherein the fragment of the first selectable marker gene encodes the carboxy terminus or amino terminus of the first selectable marker, and wherein the fragment of the first selectable marker gene is positioned adjacent to the gene of interest having the one or more mutations; and
 - (c) recombining the second nucleic acid fragment with the first nucleic acid under pressure of selection for the first selectable marker to produce a second nucleic acid molecule comprising (i) the gene of interest having the one or more mutations, (ii) a gene encoding the first selectable marker and (iii) a gene encoding the second selectable marker,whereby an allelic mutant in the gene of interest is generated.
2. The method of claim 1, wherein the first selectable marker gene having a carboxy terminus truncation or amino terminus truncation is integrated next to the gene of interest.
3. The method of claim 2, wherein the truncation is a carboxy terminus truncation and the 3' end of the gene encoding the first selectable marker is positioned on the 3' side of the gene of interest.
4. The method of claim 2, wherein the truncation is a carboxy terminus truncation and the 3' end of the gene encoding the first selectable marker is positioned on the 5' side of the gene of interest.

5. The method of claim 2, wherein the truncation is an amino terminus truncation and the 5' end of the gene encoding the first selectable marker is positioned on the 3' side of the gene of interest.
6. The method of claim 2, wherein the truncation is an amino terminus truncation and the 5' end of the gene encoding the first selectable marker is positioned on the 5' side of the gene of interest.
7. The method of claim 1, wherein the fragment of the first selectable marker gene encoding the carboxy terminus or amino terminus of the first selectable marker is located next to the gene of interest having the one or more mutations.
8. The method of claim 7, wherein the fragment of the first selectable marker gene encodes the carboxy terminus of the first selectable marker and the 3' end of the fragment of the first selectable marker gene is positioned on the 3' side of the gene of interest having the one or more mutations.
9. The method of claim 7, wherein the fragment of the first selectable marker gene encodes the carboxy terminus of the first selectable marker and the 3' end of the fragment of the first selectable marker gene is positioned on the 5' side of the gene of interest having the one or more mutations.
10. The method of claim 7, wherein the fragment of the first selectable marker gene encodes the amino terminus of the first selectable marker and the 5' end of the fragment of the first selectable marker gene is positioned on the 3' side of the gene of interest having the one or more mutations.
11. The method of claim 7, wherein the fragment of the first selectable marker gene encodes the amino terminus of the first selectable marker and the 5' end of the fragment of the first selectable marker gene is positioned on the 5' side of the gene of interest having the one or more mutations.

12. The method of claim 1, wherein the recombination is homologous recombination.
13. An isolated nucleic acid comprising a gene encoding a first selectable marker having a carboxy terminus truncation or an amino terminus truncation and a gene encoding a second selectable marker.
14. The isolated nucleic acid of claim 13, wherein at least the portion of the isolated nucleic acid containing the first selectable marker gene and the second selectable marker gene is capable of integrating into a host organism's DNA.
15. A nucleic acid comprising a gene of interest, a gene encoding a first selectable marker having a carboxy terminus truncation or an amino terminus truncation and a gene encoding a second selectable marker.
16. The nucleic acid of claim 15, wherein the truncation is a carboxy terminus truncation and the 3' end of the gene encoding the first selectable marker is positioned on the 3' side of the gene of interest.
17. The nucleic acid of claim 15, wherein the truncation is a carboxy terminus truncation and the 3' end of the gene encoding the first selectable marker is positioned on the 5' side of the gene of interest.
18. The nucleic acid of claim 15, wherein the truncation is an amino terminus truncation and the 5' end of the gene encoding the first selectable marker is positioned on the 3' side of the gene of interest.
19. The nucleic acid of claim 15, wherein the truncation is an amino terminus truncation and the 5' end of the gene encoding the first selectable marker is positioned on the 5' side of the gene of interest.

20. An isolated nucleic acid comprising a gene of interest having one or more mutations and a fragment of the first selectable marker gene which encodes the carboxy terminus or the amino terminus of the first selectable marker.
21. The isolated nucleic acid of claim 20, wherein the fragment of the first selectable marker gene encodes the carboxy terminus of the first selectable marker and the 3' end of the fragment of the first selectable marker gene is positioned on the 3' side of the gene of interest having the one or more mutations.
22. The isolated nucleic acid of claim 20, wherein the fragment of the first selectable marker gene encodes the carboxy terminus of the first selectable marker and the 3' end of the fragment of the first selectable marker gene is positioned on the 5' side of the gene of interest having the one or more mutations.
23. The isolated nucleic acid of claim 20, wherein the fragment of the first selectable marker gene encodes the amino terminus of the first selectable marker and the 5' end of the fragment of the first selectable marker gene is positioned on the 3' side of the gene of interest having the one or more mutations.
24. The isolated nucleic acid of claim 20, wherein the fragment of the first selectable marker gene encodes the amino terminus of the first selectable marker and the 5' end of the fragment of the first selectable marker gene is positioned on the 5' side of the gene of interest having the one or more mutations.
25. The isolated nucleic acid of claim 20, wherein at least the gene of interest having the one or more mutations and the fragment of the first selectable marker gene is capable of integrating into a host organism's DNA.
26. A nucleic acid that comprises a gene of interest having one or more mutations, a gene encoding a first selectable marker and a gene encoding a second selectable marker.