Chapter

Molecular Tools for Gene Analysis in Fission Yeast

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Abstract

Schizosaccharomyces pombe or fission yeast has been called micromammal due to the potential application of the knowledge derived from the yeast in the physiology of higher eukaryotes. Fission yeast has been consolidated as an excellent model for the study of highly conserved cellular processes. The possibility of using haploid or diploid strains facilitates the analysis of the dominant or recessive phenotype of an allele as well as its function, making it a model of first choice for the development of any investigation in eukaryotes cells. With a growing community that employs fission yeast as a model system for the study of numerous cellular processes, it has motivated the simultaneous development of molecular tools that facilitate the study of genes and proteins in the yeast. In this review, we present the most used molecular techniques in fission yeast for the analysis of genes, its characterization, as well as the determination of its function.

Keywords: fission yeast, gene replacement, mutants, gene expression, CRISPR/Cas, RNAi, yeast two-hybrid, microarrays, NGS, ChiP

1. Introduction

Schizosaccharomyces pombe (*S. pombe*) is a single-cell, nonpathogenic yeast, described in Germany in 1893 by P. Linder, named "pombe," and was originally isolated from East African millet beer [1]. *S. pombe* is an ascomycete fungus, whose lineage is evolutionarily remote from the yeast *Saccharomyces cerevisiae* [2]. Actually, *S. pombe* is phylogenetically as distant from budding yeasts as it is from humans. In 1950, two homothallic strains, h90 (968) and h40, and two heterothallic strains with opposite mating types, h^- and h^+, were isolated [3, 4]. There are several heterothallic strains with different genomic configurations at the mating type locus, but the heterothallic strains commonly used in the laboratory are h^{+N} (975) and h^{-S} (972) [5].

S. pombe is also called fission yeast because it is divided by binary fission. However, it has two forms of reproduction: one by binary fission and another by sporulation. Therefore, it is possible to find it in both haploid and diploid states. *S. pombe* cells are cylindrical, 3–4 μm in diameter and 7–15 μm long in haploid state, while in diploid state, they measure from 4 to 5 μm in diameter and 20–25 μm long [6]. *S. pombe* was the sixth eukaryote to have its entire genome sequenced [7]. The

genome of *S. pombe* has a size of 13.8 Mb and is organized in chromosome I of 5.7 Mb, chromosome II of 4.6 Mb, and chromosome III of 3.5 Mb, along with a mitochondrial genome of 20 Kb [8]. It contains the ribosomal RNA genes 5.8S, 18S, and 25S with a length of approximately 1.1 Mb [9]. Approximately, 4940 genes encoding proteins (including 11 mitochondrial genes) and 33 pseudogenes have been predicted. Almost 50% of fission yeast genes have at least one intron, and in total, there are 5300 introns in 2510 protein-coding genes. The process of splicing also appears to be more similar to splicing in human cells (http://www.pombase.org/status/statistics). The telomeres, centromeres, and origins of replication are more similar to complex eukaryotes than they are with the case of budding yeast. The three centromeres are 35, 65, and 110 Kb in length for chromosomes I, II, and II, respectively, with a total of 0.2 Mb [7]. The complete information of the yeast genome, gene expression data, mutations and proteins, curated literature up to tools for sequence and structure analysis is located in the main databases in the world on the NCBI, EMBL and DDBJ informatics domains as well as the Pombase database [10].

In its haploid state and in favorable conditions, *S. pombe* grows through a mitotic cycle. The optimal growth temperature for *S. pombe* cells is 30°C (25–36°C) with a doubling time of 2–4 hours [11]. In both haploid and diploid cells, *S. pombe* mitotic cell cycle is organized into the G1, S, G2, and M phases. There are two major controls regulating progress through the cell cycle: the G1–S transition and the G2–M transition. Both points are regulated by cyclin-dependent serine/threonine protein kinase Cdc2 [12, 13]. The meiotic cell cycle is a modified mitotic (M) cell cycle [14, 15]. Like other eukaryotes, in the meiotic cell cycle of fission yeast, MI is a reductional division, without intervening S phase before the second meiotic division (MII). The meiosis process concludes with the formation of an ascus including four haploid spores [15].

Under conditions of nutrient restriction especially nitrogen, cells become arrested in G1, and if the two sexual types (h^+ and h^-) are present, they will conjugate to form a diploid zygote, known as zygotic ascus [16, 17]. In a similar way as in mammals, two cells of opposite sex are recognized by the system of communication of pheromones. A cell experiment a polarized morphogenesis in the direction in the direction of the pheromones source cell by a process called shmooing. Next, two cells will merge by conjugation or "mating" producing a zygote. The zygote is diploid and could be kept in a diploid mitotic cycle if the conditions of the medium improve at this point in the cycle. If the growing conditions are unfavorable, the diploid yeast will enter into meiosis to culminate with the formation of an ascus with four haploid spores. The spores will germinate and enter again into the mitotic cycle when the environmental conditions allow it, thus closing the cycle.

S. pombe has become one of the best-studied eukaryotes today. Dr. Forsburg gave it the name of micromammal [18]. In fission yeast, genes and proteins homologous to higher eukaryotes have been described related to recombination, chromosomal organization, chromatin modification, stress response mechanisms, DNA damage response, mitosis, meiosis, cell cycle control, mRNA splicing, cell morphogenesis and polarity, and post-translational modifications of proteins such as glycosylation [19–27].

2. Gene replacement

In fission yeast, gene deletion or one-step gene deletion by gene replacement via homologous recombination is probably the most used molecular tool in the functional characterization of the function of the gene and the protein. Gene disruption is a genetic analysis strategy to achieve gene modifications, generation of

tagged protein fusions, genetic expression placed under the control of a regulated promoter, specific mutations, insertions, and deletion [28, 29]. Gene replacement by homologous recombination in *S. pombe* has allowed the construction of chromosomal interruptions of genes such as *sts1, gcs1, gsh2, hmt1* [30], and *git2/cyr1* [31].

Gene replacement requires a switch construction that contains 5′ and 3′ homologous regions of the target locus that flank a selection marker gene, and its efficiency in homologous recombination depends largely on the size of these regions [28]. The genetic construct is incorporated into the cells by transformation, then the reporter gene that will be used for gene replacement is inserted into the target gene due to the presence of terminal homologous regions in the construct, thus eliminating a large fragment of the target gene and incorporating instead, the reporter gene. At the beginning of the use of this technique, the protocol was based on obtaining the homologous regions to the target gene by digestion to flank a selection marker gene that was obtained from a plasmid containing the desired selection gene as well as the restriction sites resulting from the digestion of the homologous regions.

For this method, it was essential that DNA fragments share the restriction site for subsequent linkage. With the advance in molecular biology, methods based on PCR were developed [32]. The PCR strategy was improved by Wang et al. [32], and the protocol described the generation of construction switch gene called two-step PCR. Four oligonucleotides are required for the amplification of the homologous regions of the target gene to eliminate or modify. These PCR fragments can be called AB located in the 5′ region and CD located in the 3′ region of the target gene. A novel strategy was a little modification in the 3′ antisense oligonucleotide from AB region and 5′ sense oligonucleotide from CD region, which contains a short complementary sequence and a single restriction site to facilitate the link of the two products generated in the first PCR and then forming a product that serves as template for the second PCR. The final PCR product can be called ABCD and is cloned into a plasmid. At the same time, the gene marker selection used in the gene replacement like leu1 or ura4+ is amplified by PCR with oligonucleotides including the same restriction site in both ends, used in the 3′ antisense oligonucleotide from AB region and 5′ sense oligonucleotide from CD region of target gene to replacement. The PCR product of marker gene is incorporated into a cloning vector, and then it's digested with the unique restriction enzyme selected. Finally, the plasmid containing ABCD fragment is digested with the same restriction enzyme used to prepare the marker gene and linked to produce the AB-selection marker gene-CD gene deletion cassette.

In order to achieve the gene modification, a one-step gene deletion technique by pop-in homologous must be performed [29]. The gene deletion cassette is transformed into a yeast strain with a deletion in the endogenous gene selected like ura+ (ura4-D18) to the gene replacement [33] by the lithium acetate protocol [34]. Then, it is efficiently targeted to its homologous location in the chromosome DNA. Moreover, it is widely known that the efficiency of homologous recombination is greatly stimulated if the incoming DNA sequence has free ends. The DNA flanking to the marker gene, on each side, recombines with the genome, inserting the marker gene into the target gene, therefore disrupting or completely replacing it.

It has been reported that the optimal length of homologous sequences to achieve an efficient elimination of the gene is 80–100 pb. Nevertheless, high efficiency in mutagenesis directed for *S. pombe* has been brought, using long segments of homologia of the gene target (≥250 pb), with efficiencies in the homologous integration of up to 100% [35]. The selection marker genes used in *S. pombe* are based on gene markers of *Saccharomyces cerevisiae*. The most used genes are ura3, leu2, ade6, trp1, and his3 that synthesize enzymes used for the biosynthesis of uracil, leucine, adenine, tryptophan, and histidine, respectively [36]. A high and efficient

integration in the strains that have mutations in the locus leu1-32 and Ura4-294 of *S. pombe* with own genes *leu1*$^{+}$ and *ura4*$^{+}$ has been showed.

In addition, to make the functional analyses of various genes as well as minimize incidental recombination events between DNA sequences within the marker gene and a chromosomal sequence, gene deletion cassettes consisting entirely of heterologous DNA sequences have been designated. Those gene deletion cassettes even allow multiple gene deletions to be performed. Because the incorporation of *loxP* sites flanking the marker gene allows Cre recombinase-mediated rescue, the marker can be reused for the next gene deletion. Genes can be deleted in sequential order using different gene deletion cassettes carrying different selectable markers. Then, a gene deletion cassette would be removed from the chromosome DNA by mitotic or recombinase-mediated recombination. The strategy allows the use of the recyclable deletion cassettes, useful to disrupt the next gene of interest with the same marker gene [37, 38].

3. Heterologous protein expression

Fission yeast is a very popular system for protein expression with potential biotechnological applications. The choice of yeasts for the purification of proteins, their structural analysis, and the generation of mutants aimed at knowing the function of proteins is based on the shared conserved biological processes as cell cycle progression, protein turnover, vesicular trafficking, and signal transduction with cells of higher eukaryotes [39, 40]. In yeasts, the appropriate expression of proteins with the posttranslational modifications required allows to obtain the correct protein structure and function. So, the use of yeast in the industrial production of enzymes employed in food, medicine and health, environment, and other applications has been proposed [41, 42]. To fulfill this purpose, "humanized yeast model systems" have been created as tools to study the molecular mechanisms involved in chronic degenerative diseases such as neurological disorders [43, 44]. Due to the accessibility of the yeast to simple genetic and environmental manipulations, it reduced complexity compared to the mammalian models.

Fission yeast is an excellent system to study the complex intracellular mechanisms underlying neurodegenerative diseases such as Alzheimer's disease (AD). Heterologous expression of Tau and Aβ can provide new insights into the pathobiology of these proteins in vivo as well as the screening of compounds that may be useful in treatment and/or prevention of AD [45]. Recently, it was reported that ginger (dietary condiment) fermented with *S. pombe* had neuroprotective effects on in vivo models of AD. FG improved recognition memory, ameliorated memory impairment in amyloid beta$_{1–42}$ (A$\beta_{1–42}$) plaque-injected mice, reinstated the pre- and postsynaptic protein levels decreased by amyloid plaque toxicity, as well as attenuated memory impairment in Aβ1–42 plaque-induced AD mice [46].

Numerous expression vectors have been used in molecular studies on *S. pombe* [47–49]. A typical plasmid of *S. pombe* contains an origin of bacterial replication, an antibiotic resistance gene to select recombinant cells in bacteria, an autonomous replication sequence (ARS1), and a marker of selection of yeast. More complex plasmids can include a regulated or constitutive promoter, a transcription terminator, or epitope tags [47, 50].

The use of antibiotics to induce genes to antibiotic resistance genes as selection markers into the yeast plasmid is very frequent. The kanamycin/G418, hygromycin B, phleomycin/bleomycin, and nourseothricin/clonNat are excellent markers in fission yeast [51]. Relative to auxotrophy, new markers such as ade7, his1, his2, his3, his5, arg3, arg12, lys1, lys2, and tyr1 are being developed [51–55].

However, ade6, his3+, LEU2, and ura4+ remain the most widely used markers for the selection of multi-copy vectors in common use. The pDUAL series and pJK148 vectors have been used to achieve the conversion of the leucine auxotrophy of leu1.32 to leucine prototrophy to select integration at the leu1 locus by recombination as well as pJK210 has been used to rescue ura4.294 to target integration at the ura4 locus [36].

In regard to the promoters used in the cloning vectors to protein expression, there are many promoters between the most used such as *adh1+*, which is a constitutive promoter. The *fbp1+* is repressed by exogenous cAMP. The *SV40* promoter is of constitutive expression. The CaMV promoter is tetracycline inducible. The *inv1+* is glucose repressible. The *ctr4+* is copper repressible and *nmt1* (strong, intermediate, and weak promoters) is thiamine repressible [47]. The latter is the most used promoter and was the first characterized in the expression of protein heterologous. The *nmt1* (*no message in thiamine*) promoter (Pnmt1) is considered as an inducible/repressible strong promoter that directs the transcription. It can be repressed by the addition of thiamine to a medium or induced in the absence of thiamine [56]. Pnmt1 has excellent dynamic range and a low off-state transcription but takes 14–16 h to induce upon thiamine withdrawal. Pnmt1 responds to the lack of exogenous thiamine and is induced approximately 75-fold when thiamine is removed from the growth media. However, the activity of Pnmt1 is repressed by the yeast extract present in a medium rich in YE and YES. So, some modifications into the TATA box of Pnmt1 have been made. Variants of this promoter were developed to reduce both off-state and on-state transcription [57, 58]. Pnmt4 and Pnmt8 are excellent options to choose the desired level of expression. However, an induction of transcription of 14–16 his also required.

To solve the problem, other promoters were generated to avoid the inactivation of the promoter nmt1 in the YES culture medium. The promoters of the 276-bp eno and 273-bp gpd were modified from eno101 and gpd3 genes in *S. pombe*. Both are stronger and constitutive promoters, which increase 1.5-fold higher expression of lacZ gene than nmt1 promoter. In addition, the 276-bp eno and 273-bp gpd promoters were not affected by the components of YES medium like Pnmt1.

As it was mentioned, there are other constitutive promoters widely used in *S. pombe*. The CaMV 35S promoter is a moderate constitutive promoter in *S. pombe* derived from the native 35S promoter of the plant viral cauliflower mosaic virus through deletion of the Tet repressor [59]. The adh1+ promoter of alcohol dehydrogenase is constitutively transcribed at high levels in cells grown in glucose and glycerol. However, the adh1+ promoter is weaker than the nmt1 promoter and may only be useful if a low level of gene expression is desired [58, 60]. Padh1 has two mutant variants, namely Padh41 (a mildly weak version of the *adh1*$^{+}$ promoter) and Padh81 (a weak version of the *adh1*$^{+}$ promoter, where the TATA box sequence TATAAATA is changed into TA), and both of these promoters express the downstream gene constitutively. Padh81 has been used in the study of the dynamic of the kinetochores [61, 62].

Therefore, it is necessary to find more efficient promoters for high-expression proteins in *S. pombe*. Other induction systems have rapid response times, but have a short dynamic range or relatively high levels of off-state transcription. The lsd90 promoter that is strongly induced by heat stress was cloned into the pJH5 vector, which contains an ARS element and a truncated URA3m as selectable marker. Following the expression of the luciferase reporter into the vector and making the comparison with other promoters such as Pnmt1, Padh1, and AOX1, it was found that lsd90 promoter promotes a constitutive expression of luciferase, at a level of 19-, 39-, and 10-fold higher than the promoters above mentioned, respectively [63]. The urg1 gene was identified as a rapidly induced transcript, responding to uracil

addition in ~30 min and exhibiting low off-state transcription and high dynamic range [64] Other useful constitutive promoters in the protein expression are tif471 (with moderate force) and lys7 (weak promoter) [27, 65].

The pREP series vectors are general-purpose episomal vectors widely used in fission yeast research that contains a replication origin ARS1, ura4+, or LEU2 as the selective marker and kan, nat, hph, and bsd genes as a second type of marker of resistance to the specific antibiotics G418, clonNAT, hygromycin B, and blasticidin S, respectively. The latter are used routinely during chromosomal integration. The pREP vectors have been modified to produce novel and versatile plasmids pREP1 and pREP41. pREP1 contains a promoter derived from the gene nmt1. pREP41 contains a moderate-activity promoter (Pnmt41), whereas pREP81 contains a weaker promoter (Pnmt81). pREP vectors that contain ura4+ along with Pnmt1, Pnmt41, and Pnmt81 are named pREP2, pREP42, and pREP82, respectively [57]. The dominant selection marker genes kan, nat, hph, and bsd, which confer resistance against the specific antibiotics G418, clonNAT, hygromycin B, and blasticidin S, respectively, are used routinely during chromosomal integration [66–69].

Other important kinds of vectors of *S. pombe* are those of the pRI series generated from vector pREP, which were produced by deleting the *ars1* origin of replication sequence, and it has been used for the creation and expression of a single copy gene integrated into the chromosome [70].

The pYZ vectors are derivatives from the pREP series, which were designated for general purposes of cloning and large scale random gene cloning, as well as for allowing positive identification of cloning gene insertion and fusion to the GFP gene for analysis of gene expression. The pYZ vectors were constructed by inserting an *E. coli* α-peptide (position 239–684 on the pUC19 plasmid) of the lacZ (β-galactosidase) in opposite orientation to the Pnmt1 on the pREP series, leading to the complementation of the *lacZΔM15* deletion in *E. coli* strains such as DH5α or JM105 [56, 71, 72].

The pREP1, pREP41, pREP81, and pSGA plasmids were generated from the pREP series called pYZ1N, pYZ41N, pYZ81N (N represents an additional *Not* I site), and pYZ3N-GFP, respectively. In those vectors, the distance between the Pnmt1 and the ATG start codon remains the same as in the pREP vectors, and the promoter strength is unchanged [71]. The pYZ vectors have been useful because they were designated to produce a correct positive identification of cloning gene, fusion to the GFP, and large-scale random gene screening. The versatility of the pYZ vectors has allowed their use in numerous researches. HIV-1 vpr is a virion-associated viral protein of about 12.7 kD, whose function is required for efficient viral infection of nondividing mammalian cells such as monocytes and macrophages [73].

The HIV-1 protease (PR) is a viral enzyme encoded by vpr gene that was initially expressed in *S. pombe* from pREP1N. Vpr makes proteolytic processing required to the production of viral enzymes and structural proteins and for maturation of infectious viral particles [74].With the aim to improve the functional studies, HIV-1 *vpr* gene was cloned in the pYZ vectors. The *vpr* gene was fused to GFP in the pYZ3N-GFP vector and expressed in the yeast, where Vpr localizes to the nucleus of fission yeast cells. Expression of the *vpr* gene from the pYZ1N vector allows the analysis of the effects on cell morphology, the cell cycle G2 arrest, and cell killing [75].

In the molecular analysis of the Zika virus (ZIKV) infection, a large-scale molecular cloning and functional characterization of the viral proteins were performed. The Zika virus (ZIKV) is the causal agent of the microcephaly and the Guillain-Barré syndrome after the viral infection. However, there is insufficient knowledge about how ZIKV viral proteins are involved in cell damage. So, *S. pombe* was used to identify ZIKV factors responsible for the ZIKV-mediated cytopathic effects as well as the pathogenic factors associated with the viral infection.

By cloning the 14 coding-genes into the pYZ3N including the N-terminal GFP, it was possible to determine the subcellular localizations (nuclear, ER, Golgi, and cytoplasm) of ZIKV proteins expressed in a wild-type fission yeast strain, SP223 [70]. Importantly, seven ZIKV proteins affect cellular proliferation, which would be related to the microcephaly. So, ZIKV-induced microcephaly was proposed due to the intrauterine growth restriction, reduced cell proliferation, reduced neuronal cell layer volume, or cell death/apoptosis. Also, it was observed that prM, C, M, E, and NS4A proteins cause cell-cycle dysregulation because of cell cycle G2/M phase accumulation. These findings allow to follow the study of ZIKV infection.

Other interesting series of vectors are those that were produced as the pREP-X vectors that lack an ATG start codon [76]. Between them, pREP3X (promoter strength high), pREP41X (promoter strength medium), and pREP81X (promoter strength low), the three vectors lack tags and used Leu2 as marker. The pSLF vectors contain N-terminal or C-terminal triple hemagglutinin (3× HA) epitope tag. Between them, pSLF173 (promoter strength high), pSLF273 (medium), and pSLF373 (low), all of them contain 3xHA as tag and use ura4+ as the selective marker and the inducible promoter nmt1. From the pREP-X series were constructed several vectors with the purpose of being utilized for high-throughput functional analysis of heterologous genes in *S. pombe* such as pDS vectors that add GST taggings [50] as well as pSGA vector that includes GFP fusions.

There are many expression vectors constructed containing a destination cassette suitable for high-throughput cloning of target genes via the gateway system. There are vectors with N-terminal tagging such as the pDES173N, 273 N, and 373 N series, which add a 3XHA tag with the ura4+ gene as marker, and the vectors were constructed from the pSLF173, 273, and 373 vectors. The pDES175N, 275 N, and 375 N series add a GFP tag with the LEU2 marker, and those plasmids were built from the pSLF175, 275, and 375 vectors. The pDES177N, 277 N, and 377 N vectors add a GFP tag using ura4+ as marker selection. The pDES5XN, 45XN, and 85XN series add a RFP tag, with the LEU2 marker, which were derived from the pSLF5X, 45X, and 85X vectors. The pDES179, 279, and 379 series add a RFP tag, with the ura4+ marker, which were derived from the pSLF179, 279, and 379 vectors [77].

There are vectors with C-terminal tagging; those in the pDes173C, 273C, and 373C series add a 3XHA tag with ura4+ as marker, and the plasmids were constructed from the pSLF173, 273, and 373 vectors. The pDEs175C, 275C, and 375C series add a GFP tag with the LEU2 as marker, and those were constructed from the pSLF175, 275, and 375 vectors. The pDEs179C, 279C, and 379C series that add an RFP tag with the ura4+ marker were constructed from the pSLF179, 279, and 379 plasmids [77, 78]. These vectors exposed above lead the protein expression with N-terminal or C-terminal tagged, useful for the affinity purification or the functional analysis of target genes [77].

In 2013, an interesting series of vectors was described to PCR-based epitope tagging and gene disruption. The vectors developed were pFA6a-LEU2MX6, pFA6a-his3MX6, and pFA6a-ura4MX6. All of them were designed from the pFA6a-MX6-based plasmid (which contains antibiotic-resistance markers as kan) for amplification of gene-targeting DNA cassettes and integration into specific genetic loci, allowing expression of proteins fused to 12 tandem copies of the Pk (V5) (epitope from the P and V proteins of the paramyxovirus SV5), or 5 tandem copies of the FLAG epitope with a glycine linker. All vectors can use the LEU2, his3+, and ura4 + genes as selection markers. Also, some vectors as pFA6a-G9–5FLAG-kanMX6 and pFA6a-G11–5FLAG-kanMX6 were created, which were generated for studies of proteins when the direct epitope tagging compromises protein conformation and/or function. Other vectors were constructed to add a green fluorescent protein (GFP(S65 T)) or a monomeric red fluorescent protein (mRFP) genomic tagging as FA6A-GFP-bleMX6 [79].

Between the PK-tagging vectors are the pFA6a-6 × GLY-V5-(marker) and C-terminal FLAG-tagging vectors using KanMX6 and hphMX4 as markers. The FLAG-tagging vectors with N-terminal and C-terminal tags included the pFA6a-6 × GLY-FLAG-(maker), with kanMX6, hphMX6, natMX6, bleMX6, and his3MX6 as possible markers. Between the GFP-tagging vectors are pFA6a-GFP(S65 T)-(maker) and N-terminal and C-terminal GFP(S65 T)-tagging, which include kanMX6, hphMX6, natMX6, bleMX6, and ura4MX6. Also, some disruption plasmids as pFA6a-(maker), which has been used for gene deletions using kanMX6, hphMX6, natMX6, bleMX6, ura4MX6, his3MX6, and LEU2MX6, were constructed [79].

A novel system to cloning several DNA fragments, into a plasmid, is the Golden Gate shuffling method. Golden Gate cloning [80–82] is a modular cloning system and was set up for simultaneous overexpression of multiple genes. Some of the applications of the Golden Gate that have been tested in *Pichia pastoris* are the development of strain engineering, pathway expression, and protein production [83].

The use of this methodology for the construction of pREP1-type plasmids that expressed GOI-FPtag was reported *S. pombe*. To apply the Golden Gate cloning, several modules including promoters, tags, marker genes, terminators, and the gene of interest (GOI), which are cloned separately, are produced separately. They are digested with the enzyme BsaI that recognizes a specific sequence GGTCTC and cleaves any four-base sequence after it (such as nNNNN, mMMMM, and kKKKK) at 37°C but generates cohesive ends for various sequences. The Golden Gate method connects all the modules in the order desired in a single reaction. The cleaved fragments are joined by DNA ligase at 16°C. Once complementary four-base overhangs are connected, the site can no longer be cleaved with BsaI. The temperature shift is repeated up to 50 times until circular plasmids are efficiently produced. The system allows the assembly of up to eight expression units on one plasmid with the ability to use different characterized promoters and terminators for each expression unit [84].

In first place, modules were prepared using the pREP1 vector [70]. A segment from pREP1, which includes ars1 and Amp, was amplified by PCR with a pair of oligonucleotides containing BsaI and NotI sites. A typical expression plasmid for *S. pombe* is composed of six modules in total. The modules are a promoter, a terminator, a GOI, an FPtag fused at the N- or C-terminus, a selection marker such as an antibiotic resistance gene, and auxotrophic marker gene required to select colonies that harbor the expression plasmid. With this method, several plasmids were generated. The first plasmid was named pBMod-exv (colEI ori, Amp, ars1, NotI, and KanR sites), and this plasmid was the backbone of all vectors. Plasmids named pRGG (from pRGG-1 to pRGG-5) are expression vectors designed to express GFP-Atb2 from pREP-type multicopy plasmids. For the construction of pRGG-1, LEU2 was chosen as a marker module, whereas for pRGG-2, kan was chosen. To further demonstrate the convenience of the Golden Gate method, a series of plasmids of variable promoter strength were designed to express GFP-Atb2. The genetic elements included were the promoter (nmt1–41-81 and adh1–41-81 y urg1), an FPTag-N (GFP+ linker, mCherry+ linker, and CFP+ linker), an FPTag-C (linker+ GFP, linker+ mCherry, and linker+ CFP), GOI, and Terminator + marker (Tadh + Kan, Tadh + hpd, Tadh+nat, and Tadh+bsd) [84].

Recently, pheromone-inducible expression vectors for were developed *S. pombe*. By replacing the native Pnmt1+, the promoter regions of the sxa2+ and rep1+ genes were utilized to couple pheromone signaling to the expression of reporter genes for quantitative assessment of the cellular response to mating pheromones. The rep1+ and sxa2+ genes were chosen considering that sxa2+

mRNA increases more than 1600-fold upon pheromone perception in M-type cells [85, 86]. The EGFP open reading frame was placed downstream of the pheromone-inducible promoters, yielding pJR1-rep1-EGFP and pJR1-sxa2-EGFP, respectively [87].

In some cases of the heterologous protein expression, the better way to obtain the right protein production host is through its ability to secrete high titers of properly folded post-translationally processed and active recombinant proteins into the culture media. Proteins secreted in their native hosts will also be secreted in the culture medium. Some signal sequences used to secrete the protein into the extracellular space include α-MF and SUC2 invertase. Both are derived from *S. cerevisiae α*. α-MF is composed of a pre- and proregion and has proven to be most effective in directing protein through the secretory pathway. Other signal peptides to sorting are PHO1 P.p. acid phosphatase, SUC2 S.c. invertase, PHA-E phytohemagglutinin, KILM1 Kl toxin, pGKLpGKL killer protein, CLY and CLY-L8 C-lysozyme and syn., leucin-rich peptide, and K28 pre-pro-toxin K28 virus toxin, to produce molecules such as human interferon, α-amylase, α-1-antitrypsin, and human lysozyme [88].

One of the major problems to the correct production and purification of heterologous proteins from fission yeast is the proteolytic degradation of the recombinant gene product by host-specific proteases. To avoid that problem, a protease-deficient disruptant was constructed set by disruption of 52 *S. pombe* protease genes using the PCR-mediated single gene-targeted gene disruption method. This technique was used to delete the full open reading frame (ORF) sequence of each target protease gene, using ura4+ as the selection marker [89].

In the first place, the protease-deficient disruptant was obtained, which was amplified from genomic DNA of the *S. pombe* ARC010 strain, using appropriate adapter designed to fuse with the 5′ and 3′ termini of *ura4* (1762 bp), respectively. Then, by fusion extension PCR, *ura4* was sandwiched with the resultant PCR products to obtain the gene disruption fragment (2.2–2.3 bp). The resultant DNA fragments were then introduced into competent cells of the ARC010 strain, using the lithium acetate-based transformation method. Then, the efficient protecting activity of protease of the mutant strains was analyzed. A chromosome-integrative hGH expression vector using the pXL4 plasmid was constructed [89].

To analysed the levels of the secretory production of human growth hormone (hGH), that its known to be a proteolytically sensitive model protein. The results indicated that some of the resultant disruptants were effective in reducing hGH degradation. Although in some cases, added inhibitors of proteasas like Antipain, bestatin, Chymostatin, E-64, Leupeptin, pepstatin, Phosphoramidon, EDTA, aprotininto avoid protein degradation were necessary. Eight protease coding genes useful for reducing degradation of recombinant proteins [isp6 (subtylase type 9 proteinase), pgp1 (endopeptidase), psp3 (subtylase type peptidase), sxa2 (serine carboxypeptidase), ppp51 (aminopeptidase), ppp53 were identified (zinc metallopeptidase), ppp60 (metalloprotease) and ppp80 (peptidase)], the use of a strain lacking the aforementioned enzymes allowed a high level of recombinant hGH production. This publication raised the need to evaluate different proteases to identify those that are the best candidates for the production of recombinant proteins, as well as for functional screening, specification, and modification of proteases in *S. pombe* [89].

In relation to the methods for the transformation of *S. pombe*, the lithium acetate and polyethylene glycol-based transformation of plasmid DNA are the most popular and temperature stresses. With these methods, it is possible to achieve transformation efficiencies between 1.0×10^3 and 1.0×10^4 transformants per microgram of the plasmid with 10^8 *S. pombe* cells [90, 91].

4. Mutants to analyze the function of genes

The use of mutants to analyze the function of genes has been a tool widely used in *S. pombe*. In this yeast, several types of mutants have been produced such as the temperature-sensitive mutants with conditional defects in the ability to participate in some cellular process in the cell cycle, cytokinesis, lipid metabolism, or DMSO-sensitive [92]. The use of temperature changes to impose a restrictive condition is a strategy widely employed. But, there are methods such as altered sensitivity to drugs, pheromones, and changes in ionic strength, among others. For mutational analysis, the haploid state offers the advantage to observe the effect of specific mutations [93].

In the case of the essential genes, a lethal phenotype is frequently observed. To achieve the study of essential genes, there are two strategies. First, the mutations or gene deletions are created in the diploid state and then the synthetic lethality is studied in the haploid state. Sometimes, it's possible to observe a slow-growth phenotype, in which haploid cells can partially survive without function of the inactivated gene. Second, the creation of the conditional lethal mutations allows to study a relatively normal gene function under permissive conditions, and then the loss of function is observed under nonpermissive conditions. The most used conditional mutants are the temperature sensitivity, sensitivity to DNA-damaging agents, sensitivity to drugs and inhibitors, and dependence on amino acids or certain carbon sources for viability. Three methods highly used to produce mutants are gene knockouts, random mutagenesis, and site-directed mutagenesis [94].

5. CRISPR/Cas9

The CRISPR/Cas system is a bacterial defense mechanism, and its main function is to identify and degrade exogenous nucleic acid sequences [95]. CRISPR-CAs is organized in an operon, which codes the CAS proteins, and a series of identical repeated sequences separated by other sequences known as spacers, which are recognized by intruding DNA molecules [96]. A part of the nucleic acid stranger is incorporated into the spacer's zone of the operon using the Cas proteins, which degrade the strange DNA. Next, the transcription of CRISPR-Cas generates a precursor CRISPR-RNA or pre-crRNA, which is then processed to generate crRNAs of small size, which are complementary to the sequence of the foreign DNA. In the last known phase of interference, Cas proteins, using as a guide to crRNAs, detect intruding sequences and degrade them [96].

The CRISPR/Cas technology allows to identify a specific segment of DNA, remove, or replace it using always the same tools: a duplex RNA with the copy of the DNA to be identified (sgARN) and a short sequence adjacent to the proto-spacer (PAM) that will bind to DNA and stabilize the protein Cas9, protein with endonuclease activity, and helicase guided by the sgARN that separates and cuts the two strands of DNA. A Cas9-gRNA plasmid expressing the active Cas9 enzyme and sgRNA, as well as another plasmid with donor DNA for each deletion are required. The CRISPR-Cas technology allows targeting of multiple genetic manipulations to the same strain, it avoids indirect physiological effects, and it limits the perturbation of the local chromatin and transcriptional environment to the gene manipulation of interest. In fission yeast, this technique has allowed to produce genetic modifications as point mutation knock-in, endogenous N-terminal tagging, and genomic sequence deletion [97].

Recently, a web-tool called CRISPR4P CRISPR for *Pombe* or CRISPR *Pombe* PCR Primer Program was developed as freely available from the website (bahlerlab.info/crispr4p) [98]. This tool was created to support the design of all kinds of primers required for the deletion of any genomic region: PCR-based sgRNA cloning, PCR-based synthesis of DNA template for the deletion by homologous recombination, and checking primers to confirm the deletion. Through CRISPR/Cas9-based approach in *S. pombe*, the success in the deletion of over 80 different noncoding RNA genes that were lowly expressed was reported. Using the web tool, the preparation of G1-synchronized and cryopreserved *S. pombe* cells was achieved, whose major property was the efficiency and speed for transformations. The steps to achieve the deletions reported by Rodríguez-López et al., 2016, are: (1) identify better sgRNAs to target region of modification using CRISPR4P tool. (2) Design primers required for whole process using CRISPR4P including sgRNA cloning; synthesis DNA template for homologous recombination (HR template) for gene deletion; and check primers to confirm gene deletion. (3) Clone sgRNAs into nourseothricin-selectable plasmid pMZ379 that contains Cas9 enzyme gene, the *natMX6* selection marker, and the *rrk1* promoter/leader. (4) Produce the HR template by PCR using primers with sequences flanking the region of modification (deletion) and overlapping at their 3′ ends. (5) Delete region of interest by co-transforming sgRNA/Cas9-plasmid and HR template into *S. pombe* cells, previously synchronized and cryopreserved to increase transformation efficiency [99].

A gap-repair-based CRISPR/Cas9 procedure allows to efficiently knockin a point mutation in fission yeast. The rpl42-P56Q mutation confers cycloheximide resistance (CYHR) [100]. Employing this technique, a CCC codon for proline was changed, and with the use of a pair of 90-nt complementary oligos as donor DNA, the gap repair procedure achieved a high editing efficiency (84%).

Using the CRISPR-Cas9, yeast strains, functional and successfully complemented with the markers ura4-D18, leu1-Δ0, his3- Δ0, and lys9-Δ0, were created. To achieve the goal, all the components were assembled with the "BsaI-pad," a single 42 bp region containing two BsaI cutting sites to produce the plasmids pYZ182, pYZ183, and pYZ184 with nmt1, nmt41, and nmt81 cassettes, respectively. Using that design, the marker genes ura4, leu1, his3, and lys9 were integrated separately. Later, the plasmids were transformed into yeast [101].

Recently, the type VI CRISPR system, Cas13a from Leptotrichia shahii (LshCas13a), was employed to introduce genetic changes on the DNA, disrupting or editing to target and knockdown endogenous gene transcripts with different efficiencies in *S. pombe* [102].

6. RNAi

RNA interference (RNAi) is a highly conserved eukaryotic gene regulatory mechanism, which uses small noncoding RNAs to mediate posttranscriptional gene silencing as a host defense mechanism. It was described that *S. pombe* has the entire RNAi machinery (Dcr1, DICER ribonuclease; the Rdp1, RNA-dependent RNA polymerase 1; and the Ago1, Argonaute family member). In *S. pombe*, the role of the RNAi pathway on the heterochromatin assembly has been widely studied [103]. RNAi plays a role in regulating expression of Tf2 retrotransposons, and it is also involved in the RNAi-dependent heterochromatin assembly by the Hsps, Hsp90 and Mas5 (a nucleocytoplasmic type-I Hsp40 protein).

siRNA is generated by the Dicer family endoribonuclease Dcr1, from double-stranded noncoding RNA that is complementary to heterochromatin. The siRNA

duplex is loaded onto a non–chromatin-associated complex called Argonaute, small interfering RNA chaperone (ARC), which contains the Ago1 endoribonuclease. The loading of the siRNA duplex onto the Ago1 subunit requires the two ARC-specific subunits, Arb1 and Arb2, which also inhibit the release of the passenger strand [104]. Thus, this complex changes its subunits' composition to form a chromatin-associated effector complex called RNA-induced transcriptional silencing (RITS) [105]. The RITS complex is composed of Ago1, now binding single-stranded siRNA as a guide for target recognition, and the two RITS-specific subunits: Chp1 and Tas3. Chp1 uses a chromodomain to recognize H3K9me, whereas Tas3 bridges Ago1 and Chp1 [106].

To analyze the role of the RNAi in fission yeast, the lacZ fission yeast system was employed. With this system, it was possible to know that the gene inhibition is dependent on the dose of the antisense RNA, the size of the antisense transcript, as well as the targeted region. Any of them can affect the efficacy of target gene inhibition. The generation of dsRNA through either intermolecular or intramolecular hybridization is central to make the antisense RNA-mediated gene silencing in *S. pombe* [107]. As a genetic tool to analyze the function of genes, the ura4-based RNAi-based selective assay was developed using a repressible thiamine promoter [108]. The RNAi must be optimized in order to know the minimum requirements to achieve the knockdown of a specific gene. U-HP construct was produced as a hairpin complementary to 200 bp of ura+ gene expressed from the nmt1 promoter and integrated at ars1 on chromosome 1. U-HP silences ura4+ inserted nearby to centromere 1, but not the endogenous ura4+ gene. Interestingly, in *S. pombe*, exogenous siRNAs can only silence efficiently in *trans*, when the target locus is near endogenous sites of heterochromatin.

An interesting proposal to analyze the role of the siRNAs in *S. pombe* was achieved with the development of a GFP-HP construct. This system was generated under control of the Pnmt1, and it contains two GFP open reading frames arranged in an inverted orientation, around the first intron from the rad9 gene. When it was probed, it was demonstrated that GFP-HP induces trans-silencing of target genes. GFP siRNAs generated by the expression of a GFP-HP can act in *trans* to establish heterochromatin on target genes bearing homology to GFP siRNAs and silencing their expression. This silencing does not require other manipulations, such as deletion of eri1+ or increased expression of Swi6HP1, a heterochromatin component, to promote RNAi-mediated silencing in *trans* [109].

7. Yeast two-hybrid system

The yeast two-hybrid system (Y2H) is a method widely employed to study the physical interaction of proteins by the downstream activation of a reporter gene. Considering that many eukaryotic transcription factors are organized in a modular way with at least two domains, it is possible to separate them into their domains [110].

In this assay, two plasmids are created; the first is named the bait plasmid including the DNA-binding domain of a transcription factor joined to one of the proteins to analyze and it is named Bait. In this vector, a selection marker is included such as HIS3, ADE2 (Gal4 system), or LEU2 (LexA system with binding sites for the DNA-binding domain). The second vector is named prey including the activation domain of the transcription factor joined to the second protein to study in the interaction, named Prey. As in the other vector, a different selection marker is included. When the Bait and Prey proteins are put together by protein interaction, they restored the organization of the transcription factor, and then

they can activate the transcription of the reporter gene as the *E. coli lacZ* gene. The transcription factors more frequently used are *Escherichia coli* LexA protein and the yeast Gal4 protein, as well as herpes simplex virus VP16 protein and the B42 acid blob from *E. coli* [111].

Gal4 is a transcriptional activator in yeast that binds to UAS (upstream activation domain), a specific DNA sequence, and activates transcription in the presence of galactose. The separation of Gal4 in two fragments produces N-terminal DNA-binding domain (DBD) and C-terminal transcriptional activation domain (AD), but did not activate transcription in the presence of galactose until both domains are associated to reconstitute a fully functional Gal4. Some disadvantages of the assay consider that in some cases, it's necessary to modify the bait proteins because a protein with both DNA-binding and transcriptional activating properties is possible to be found. Some fused proteins may not be able to enter or be expressed in the yeast nucleus. The GAL4 BD has its own nuclear localization signal (NLS). If the GAL4-based Y2H system fails, the interaction could be analyzed and detected successfully using a LexA-based Y2H system [110, 111].

The Y2H system has been widely used. In *S. pombe*, its use in the searching of the new determinants of aging was reported. Chen et al. described a method to select long-lived mutants from *S. pombe* bar code-tagged insertion mutant library (each insertion had a unique sequence tag called a bar code produced by random barcode). With this strategy, it was possible to identify an insertion mutation or deletion in the cyclin gene *clg1+* that extended the chronological aging of the yeast. At the same time, it was determined that depletion of Clg1p also decreases the cyclin-dependent kinase Pef1p and an extended longevity was observed. To analyze if the phenotype was produced by direct or indirect contact, a yeast two-hybrid analysis and immunoprecipitation assay were performed [112].

To the assay, the entire *pef1+* ORF was fused to the Gal4p DNA-binding domain and the entire *clg1+* ORF was fused to the Gal4p activation domain. A physical interaction was observed between Clg1 and Pef1. To perform this assay, the pGBT9-Pef1 and pGAD424-Clg1(full length) or pGAD424-Clg1(1–590) plasmids were constructed and transformed into the *Saccharomyces cerevisiae* two hybrid indicator strain Y187 (*MAT*α, *ura3–52*, *his3–200*, *ade2–101*, *trp1–901*, *leu2–3112*, *gal4*Δ, *met-*, *gal80*Δ, *MEL1*, and *URA3*::*GAL1UAS-GAL1TATAlacZ*, Clontech). Positive transformants were selected on complete medium plates without leucine and tryptophan at 30°C for 3 days. The reporter gene *lacZ* expression was probed from five individual colonies from each transformation and was patched on plates that require both plasmids for growth and incubated at 30°C for 2 days. Then, the coimmunoprecipitation was performed with FLAG-tagged Clg1p, which was expressed in cells that also expressed triple HA (3HA)-tagged Pef1p [113]. Using Western blotting of FLAG-Clg1p immunoprecipitates revealed the presence Pef1p-3HA. Chen et al. concluded that Clg1p interacts with the cyclin-dependent kinase Pef1p in *S. pombe* cells. In addition, a third Pef1p cyclin named Psl1p was identified. Genetic and coimmunoprecipitation assays indicated Pef1p controls lifespan by downstreaming the protein kinase Cek1p [114].

8. DNA microarray

DNA microarray is an orderly set of segments of genes that are immobilized on a surface called chip. The DNA arrangements allow the massive study of the gene expression of an organism, and it allows to know the differences of gene expression between two samples of RNA in a given cellular condition. In cells that present some mutation or elimination in some genes or cells derived from individuals

with some infectious disease or not, the microarrays allow the identification of sets of genes related to the gene or genes under study or the condition of disease. Comparing RNA prepared from diseased cells and normal cells can lead to the identification of sets of genes that play key roles in diseases. Genes that are overexpressed or underexpressed in the diseased cells often present excellent targets for therapeutic drugs.

The application of DNA microarray technology requires a genomic library conformed by a set of DNA segment derived from each of the genes of the model of interest, which is generated from PCR products or synthetic oligonucleotides, as well as the design and construction of the arrangement, to determine the physical location and accurate identification for the analysis and interpretation of gene expression data. Microarray analysis requires total RNA extraction from control and the problem obtained by any strategy optimized for certain cell type [115]. Total RNA control and the problem should be submitted to retrotranscription incorporating uracil marked with a fluorescent molecule as dUTP-Cy3, dUTP-Cy5, dUTP-Alexa 555, dUTP-Alexa 647, and biotin, among others. The labeling of the cDNA must be differentiable between the two tissues to be analyzed [116]. The hybridization of the microarray containing probe sets that represent a finite number of transcripts is carried out. Fluorescence reading is obtained with a microarray reader. The quantification of the signal produced by the fluorescence of the spots allows to calculate for each point the mean density value of the nucleotides marked cDNA (g. e. of Alexa555, Alexa647) and the average value of the background. To identify the genes expressed differentially in the experiment, it is necessary to perform a statistical analysis, from the normalization of the data. The goal is to analyze those genes that move away from normalization through the value of Z [117]. The genes with the value of $Z > 2$ present a statistically significant change between the experimental condition and the control (genes with greater or lesser expression). [116]. Easy and useful software for data analysis of microarrays is GenArise (computer unit of the Institute of Cellular Physiology of UNAM (http://www.ifc.unam.mx/genarise/).

From the data that record a significant change, it is necessary to determine its association to some biological processes by clustering analysis for gene expression [118].

With this molecular tool, it was possible to analyze in fission yeast the effect of Spc1, a mitogen-activated protein kinase in the stress responses. Spc1 is an activator of transcription factors that control gene expression in response to extracellular stimuli and is also known to interact with the translation machinery. Using microarrays of Affymetrix GeneChip Yeast Genome 2.0 Array, it was possible to know the set of genes that is regulated by SPC1, and this analysis was carried out without and with a stress condition to evaluate the effect of the wild-type SPC1 kinase and Spc1K49R, a mutant of this enzyme. Spc1 and Spc1K49R were separately overexpressed in *S. pombe* cells, and gene expression was compared with the control cells (which are transformed with the empty with the Pnmt1). Interestingly, only 42 genes were found with differential expression after Spc1 overexpression, while 132 genes were found to be differentially expressed after Spc1K49R overexpression. Some of the genes up-regulated after Spc1 overexpression were Mitogen-activated protein kinase sty1 and M cell-type agglutination protein mam3. The downregulated genes were NAD-dependent malic enzyme, meiotic cohesin complex subunit Rec8, and aph1 bis(5'-nucleosidyl)-tetraphosphatase. Between genes differentially expressed after Spc1K49R overexpression, those upregulated included pheromone p-factor receptor, RNA-binding protein involved in meiosis Mei2, MAP kinase Spk1, cell agglutination protein Mam3, M-factor precursor Mfm1, and M-factor precursor Mfm3. And some downregulated were serine/threonine protein kinase Gsk3, RNA-binding protein Sap49, and Argininosuccinate lyase [119].

In 2016, the role of the putative NO dioxygenase SPAC869.02c (Yhb1) and the S-nitrosoglutathione reductase Fmd2 was analyzed. Both proteins are NO-detoxification enzymes. In the study, it was found that exogenous NO protects *S. pombe* cells against H_2O_2-induced oxidative stress by inhibition of $Fe^{(3+)}$ to $Fe^{(2+)}$ conversion, upregulation of the H_2O_2-detoxifying enzymes, as well as downregulation of the MRC genes. Transcriptomic analysis was carried out with an Affymetrix Gene Chip Yeast Genome 2.0 Array [120].

The fission yeast *S. pombe* generally reproduces by mitosis. To know the role of the fhl1 protein in meiosis, a microarray analysis of the fhl1Δ strain was performed. Interestingly, it was found that nitrogen starvation-response genes are controlled by fhl1. Some of them are genes of mating and sporulation such as isp4, mfm1, mfm2, Mat-Mc, ste4, ste11, map1, map3, mei2, and mcp7 [121].

9. Next-generation sequencing

Next-generation sequencing (NGS) involves the parallel mass sequencing of thousands of DNA fragments. Sample processing for NGS can be summarized as follows: First, nucleic acid extraction (DNA or RNA). Second, selection of the type of NGS sequencing (targeted sequencing, whole exome sequencing, and whole genome sequencing). Third, library generation by DNA fragmentation, ligation of adaptors, and amplification and sample enrichment. Fourth, template generation or cluster generation according to the platform of sequencing. Fifth, sequencing (using a specific platform as Illumina, PacBio). Sixth, data analysis. Data analysis includes the quality evaluation of the sequence, alignment to reference sequence to identify some possible variations such as single nucleotide polymorphism (SNP) or insertion-deletion (indel) identification, phylogenetic or metagenomic analysis, as well as the identification, interpretation, and classification of pathogenic variants [122, 123].

Splicing is an essential step in eukaryotic gene expression. Introns are excised by the spliceosome, composed of five uridine-rich small nuclear RNAs (U1, U2, U4, U5, and U6 snRNAs) and several polypeptides. To characterize the U2·U5·U6 complex of *S. pombe*, cell lysates were obtained. A large-scale isolation of the U2·U5·U6 complex was performed using double-affinity purification using a split TAP-tag approach [124], with protein A attached to U2 snRNP protein Lea1 (U2 A′ in humans) and calmodulin-binding peptide (CBP) attached to U5 snRNP protein Snu114 (U5 116K in humans). After the purification of the complexes, the content of protein and RNA associated to the U2·U5·U6 complexes was analyzed. By denaturing PAGE and high-throughput sequencing (RNAseq), the presence of U4, U1, and heterogeneous higher molecular weight species was shown. In addition, the U2·U5·U6 snRNA complex contains excised introns, indicating that it is primarily the ILS (intron lariat spliceosome) complexes. The protein content of the ILS complex of *S. pombe* was similar to the spliced product of humans and the ILS complexes assembled on single pre-mRNAs in vitro from *Saccharomyces cerevisiae* [112].

There are some other techniques to study several aspects of the physiology of *S. pombe*. Chromosome conformation capture (Hi-C) is a technique widely used to identify long-range chromatin interactions. The spatial organization of mitotic chromosomes with the greatest compaction during mitosis is an interesting aspect of the cell cycle. In *S. pombe*, it is known that condensin, a structural maintenance of chromosomes (SMC) family member, has a role on the chromatin architecture. Biochemical studies have been applied to discover the more relevant points of the mechanism. By chromosome conformation capture (Hi-C), it was demonstrated that condensin is able to replace short-range local contacts in the interphase with longer-range interactions in the mitosis. Condensin achieves this by setting up

longer-range, intrachromosomal DNA interactions, which compact and individualize chromosomes. Even local chromatin contacts are constrained by condensin during mitosis [125].

Finally, it is necessary to mention that Rallis & Bähler offered to the world pombe community an excellent review showing the relevance of *S. pombe* in the eukaryotic studies employing a wide genome screen and phenomic assays, ranging from growing conditions to metabolomics [126, 127].

10. Conclusion

Schizosaccharomyces pombe is an excellent model to study highly conserved processes between eukaryotes, its versatility, ease of manipulation, its accessibility to genetic manipulations, making it a great model system increasingly used by a growing scientific community interested in fission yeast. At the same time, this interest has promoted the technological development, the implementation, and the continuous improvement of new molecular tools that when applied to *S. pombe* will allow to elucidate new mechanisms of cellular processes with potential application to the Eukaryotic kingdom including the human being.

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