**File 1 Landing site CHRIII.xlsx**

*This file contains the raw data for Table 4 and Figure 3.*

Sheet chromosome III - This data file contains an overview of the genes encoded on SeScCHRIII, their location, their orientation, systematic gene name and functional gene name. The ORFs were screened for function and genes with an non-essential cellular function were highlighted in green and marked as potential landing site. Autonomously replicating sequences (ARS, grey), the centromere (grey) and delta sequences (orange) are highlighted in orange.

CDS\_CBS1483\_Seub03-Sc03\_NonCodi – This sheet gives the locations of non-coding sequences on SeScCHRIII. The coding sequences flanked by 800 nucleotides upstream and 300 nucleotides downstream each ORF, corresponding to the promoter and terminator sequences, were subtracted from the whole chromosome sequences, leaving the coordinates of non-coding sequences.

Sheet mummer\_200nt\_markers – The non-coding sequences from the sheet ”CDS\_CBS1483\_Seub03-Sc03\_NonCodi” were used to make k-mers of k = 200 nucleotides. The resulting k-mers were used in a mummer alignment to the genome sequence of CBS 1483. K-mers that have only one hit [% IDY = 100] when aligned to the whole genome were highlighted in yellow and regarded as unique in CBS 1483.

Sheet mummer\_500nt\_markers - The non-coding sequences from the sheet ”CDS\_CBS1483\_Seub03-Sc03\_NonCodi” were used to make k-mers of k = 500 nucleotides. The resulting k-mers were used in a mummer alignment to the genome sequence of CBS 1483. K-mers that have only one hit [% IDY = 100] when aligned to the whole genome were highlighted in yellow and regarded as unique in CBS 1483.

Sheet Selected genes and sites – This overview contains the genes and unique k-mers for potential landing sites. The selected genes and sites are highlighted in green. Sites highlighted in yellow should not be regarded as potential landing sites due to their function for replication.

**File 2 Genome editing efficiencies.xlsx**

*This file contains the raw data for Figure 1C, Figure 4B, Figure 5C and Figure 8.*

Sheet LOH - Editing efficiencies in CEN.PK113-7D and CBS 1483 were calculated from colony counts with and without reporter gene (ymNeongreen fluorescence) from three biological replicates. Diagnostic PCR specifications per landing site are indicated.

Sheet CBS1483 – Editing efficiencies in CBS 1483 were calculated from colony counts with and without reporter gene (ymNeongreen fluorescence) from four biological replicates. Diagnostic PCR specifications per landing site are indicated.

Sheet WS3470 - Editing efficiencies in WS34/70 were calculated from colony counts with and without reporter gene (ymNeongreen fluorescence) from three biological replicates. Diagnostic PCR specifications per landing site are indicated.

Sheet in vivo assembly - Editing efficiencies for in vivo assembly of two fragments (ymNeongreen and Scarlet) were calculated from FACS screening and gating of the fluorescent populations from three biological replicates. Diagnostic PCR specifications are indicated.

**File 3 Fermentation landing sites characterisation.xlsx**

*This file contains the raw data for Figure 6.*

This file contains the raw data of the fermentation in septum flanks with strains that have ymNeongreen integrated into the respective landing sites. The “overview” sheet contains the fermentation specifications, including wake up cultures, pre cultures and fermentation conditions. The “OD660” sheet contains the OD660 measurements over time per strain in biological triplicates and technical duplicates. Averages of the OD660 measurements over time are plotted with the standard deviations. The “HPLC” sheet contains the HPLC measurements of the sugars (maltotriose, maltose, glucose and fructose), ethanol and glycerol in biological triplicates and technical duplicates. Averages of the metabolites are plotted over time with the standard deviations. The “GC” sheet contains the GC measurements of the ketones (2,3-diacetyl and 2,3-pentadione) and esters (Ethyl acetate, isobutyl acetate, ethyl butyrate, isobutanol, isoamyl acetate, isoamyl alcohol, ethyl hexanoate, ethyl octanoate, ethyl decanoate) in biological triplicates. Averages of the metabolites are plotted over time with the standard deviations.

**File 4 Fermentation ALDC.xlsx**

*This file contains the raw data for Figure 7.*

This file contains the raw data on the septum flask fermentation with the strains containing an acetolactate decarboxylase gene.

The sheet “Epx data” contains the fermentation specifications. The sheet “Inocul. List Erlenmeyer fl” contains the information on the wake up cultures, pre cultures and fermentation culture transfers. Also, the OD660 measurements of the fermentation are collected in this sheet. The “OD660” sheet contains the OD660 measurements over time per strain in biological triplicates and technical duplicates. Averages of the OD660 measurements over time are plotted with the standard deviations. The HPLC sheet contains the HPLC measurements of the sugars (maltotriose, maltose, glucose and fructose), ethanol and glycerol in biological triplicates. Averages of the metabolites are plotted over time with the standard deviations. The “GC\_Ketones” sheet contains the GC measurements of the ketones (2,3-diacetyl and 2,3-pentadione) in biological triplicates. Averages of the metabolites are plotted over time with the standard deviations. The sheet “Plots” contains all the plots relevant for the fermentation experiment.

**File 5 Overview pastorianus strains CHRIII.xlsx**

*This file contains the raw data for Figure 5A.*

This file contains the numerical values of the CNV analysis of chromosome III configurations evaluated in 47 *S. pastorianus* strains that were deposited at the NCBI database.

**File 6 CNPlotAll\_SRA\_final\_median.pdf**

*This file contains the raw data for File “File 5 Overview pastorianus strains CHRIII.xlsx”.*

This file contains the CNV plots for chromosome III configurations evaluated in 47 *S. pastorianus* strains that were deposited at the NCBI database. This file contains the information for the analysis performed in “File 5 Overview pastorianus strains CHRIII.xlsx”.