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Versatile Extensions of the Flp-Recombinase-Mediated Cassette Exchange Technology: RMCE multiplexing approaches meet the needs for predictable genome engineering

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Titel der Dissertation: Die Erweiterung des Flp-Rekombinase vermittelten Kassettenaustausch „Multiplexing RMCE“ erlaubt die zielgerichtete Modifikation von mehreren genomischen Loci.

Zusammenfassung

Ortsspezifische Rekombination hat sich während der letzten 20 Jahre zu einem wichtigen Werkzeug für die Modifikation des Mammalia-Genoms entwickelt. Neben einer Vielzahl von ortsspezifischen Rekombinasen, bilden die Cre/loxP- und Flp/FRT-Tyrosin-Rekombinasen die effizientesten Systeme für die zielgerichtete Modifikation von Zelllinien und embryonalen Stammzellen. Flp-Rekombinase vermittelter Kassettenaustausch (RMCE) erlaubt sukzessive zielgerichtete Austauschreaktionen von Sequenzabschnitten an einem prä-charakterisierten Locus. Das Kerngerüst des Mechanismus bilden zwei heterospezifische FRT Sites, die nur homospezifische (identische) Sites erkennen aber nicht mit heterospezifischen Sites kreuzrekombinieren. Für die Austauschreaktion wird ein Überschuss an Donorplasmid und Flp-Rekombinase in die Zielzelle transfiziert, die bereits mit einer genomisch verankerten Austauschkassette versehen ist. Andere Techniken zur zielgerichteten Modifikation von eukaryotischen Genomen (Flp-in) haben den Nachteil, prokaryotische Sequenzen oder Selektionsmarker, die Silencing-Effekte hervorrufen können, permanent zu integrieren.

Multiplexing-RMCE wurde entwickelt um mehrere unabhängig voneinander ablaufende Austauschreaktionen in einer Zelle zu ermöglichen. Zur Umsetzung dieses Systems wurden sieben neue mutierte FRT-Sites konstruiert und auf Selbsterkennung mit identischen Sites und Kreuzrekombination mit heterospezifischen Sites überprüft. Erkennungssequenzen, welche diese Kriterien erfüllten, wurden als RMCE-kompatible Paare zusammengestellt und auf die Fähigkeit zum transienten Kassettenaustausch in der Fibroblastenzelllinie NIH/3T3 überprüft. Von drei Paaren, die aus den neuen FRT-Sites gebildet wurden, konnten die Kombinationen der mutierten FRT Sites F_{14} - F_{15} sowie F_{13} - F_{14} neben der bereits etablierten Kombination F_3 - F_{wt} die besten Resultate erzielen. In einem finalen Experiment konnten zwei parallel ablaufende Austauschreaktionen auf genomischer Ebene mit den FRT-

Paaren F_3 - F_{wt} und F_{13} - F_{14} in NIH/3T3 Zellen molekularbiologisch nachgewiesen werden.

In der Biotechnologie könnte das neue System für die unabhängige Expression von Antikörperketten, T-Zellrezeptoren oder anderen Proteinuntereinheiten eingesetzt werden. Die Verfügbarkeit von neuen heterospezifischen FRT-Sites erlaubt die Entwicklung von neuen Strategien für die Modifikation von größeren Gendomanen. Durch die Optimierung des Gentransfers in Primärzellen, embryonalen oder induzierten pluripotenten Stammzellen kann der (erweiterte) RMCE auch für diese Systeme in medizinisch relevanten Feldern Anwendung finden.

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Title of dissertation: Versatile Extensions of the Flp-Recombinase-Mediated Cassette Exchange Technology: RMCE multiplexing approaches meet the needs for predictable genome engineering

Summary

In the recent 20 years, site specific recombination has become a wide spread tool for the precise modification of the mammalian genome. Among a variety of site specific recombinases the tyrosine recombinase systems Cre/loxP and Flp/FRT were shown to be the most efficient for targeted modification of mammalian cell lines and murine embryonic stem (ES) cells. Flp-Recombinase-mediated cassette exchange (RMCE) allows several successive targeting actions addressing a pre-defined genomic locus. It applies heterospecific FRT sites, which can recognize and recombine with homospecific (identical) partner sites but do not allow cross-interaction with heterospecific partners. Thus, it allows targeted integration of sequences of interest into a characterized genomic locus, delivered by a donor plasmid and a Flp-recombinase expression unit. Unlike related targeting techniques such as Flp-in, RMCE overcomes silencing as the procedure does not co-introduce plasmid sequences. Moreover, it does not leave behind a selection marker, which would be another potential source of silencing.

Multiplexing-RMCE was developed to permit several independent exchange reactions in a given cell. To implement this system seven novel heterospecific FRT mutants have been designed, which were subsequently characterized for two critical parameters. An interaction assay for two homospecific FRT sites allowed determining self-recognition potential, which should be comparable with the FRT wild-type site. A second test evaluated cross-recombination between two heterospecific FRT sites, which was necessary to avoid excision, inversion or translocation of the flanked cassette. Three pairs of novel FRT sites, which complied with these criteria, were selected to investigate their ability for cassette exchange, first in a transient system. Next to the reference pair F_3 - F_{wt} , the combinations F_{13} - F_{14} and F_{14} - F_{15} turned out to be the best candidates. In the ultimate setup, the feasibility of these novel sites in a genomic based multiplexing-RMCE assay could be demonstrated to act in parallel to

the conventional combination F_3 -F. In detail, two genomic loci F_3 -F and F_{13} - F_{14} could successfully be subjected to simultaneous Flp-mediated genomic targeting (“multiplexing”) reactions in NIH/3T3 cells.

Potential applications of the system in Biotechnology and Cell Biology are the independent expression of antibody chains at a given ratio. Applications can be extended to T-cell receptors or other proteins that are composed from two or more different subunits.

Without doubt, the emergence of novel heterospecific FRTs will enter sophisticated gene targeting approaches, for instance the sequential elaboration of large gene domains in cell lines. With the advent of new transfection or transduction options, primary cells and induced pluripotent stem (iPS-) cells will be other targets of choice for an expanded RMCE concept.

1 Introduction

During the last decades, the genetic research community has developed a great variety of tools to identify, isolate, amplify, analyze and express genes *in vitro* and *in vivo*. For most of these tasks, these genes have to be transferred into prokaryotic or eukaryotic organisms. The transfer of genes into eukaryotic cells, for instance by episomal plasmids or viruses is in general accompanied by modification of the genome, due to stable integration of the vector DNA into the host cell genome. Manipulation of the genome has a high importance for several fields as gene therapy or the development of gene 'knock out' mutants by gene targeting approaches. The creation of those mutants required the discovery of cellular machineries, which are able to cognize specific stretches of DNA sequences to allow specific manipulation of the genome (homologous recombination or site specific recombination, see below).

1.1 Recombination

As mentioned above, one way of DNA manipulation is the rearrangement of DNA sequences (recombination). This process can be divided into cut followed by paste (rejoining) of DNA. Besides mutation, recombination is the second process, which is essential for genetic variability in a population. Recombination is present in all life forms and is grouped into three subtypes: homologous recombination (HR), non-homologous recombination (illegitimate recombination) and site specific recombination (SSR).

1.2 Homologous recombination

Homologous recombination (HR) is a natural process found in all life forms. It promotes genetic diversity and is therefore a key player of evolution. It presents a repair mechanism for DNA double strand breaks (DSB) and serves to remove damaged or mutated DNA by replacing it with non-mutated DNA from a homologous sister DNA sequence. Prerequisites for homologous recombination are two extended (several kilobase (kb)) stretches of identical double stranded DNA, flanking the damaged DNA

sequence. Homologous recombination precisely cuts and pastes DNA without introduction or removal of foreign DNA into the damaged locus. This process is monitored by the appearance of crossovers and Holliday junctions at the point of DNA breaking and rejoining (Haber *et al.*, 2004). Enzymes (recombinases) involved in that process do not bind to specific DNA motif sequences but at some unspecified position in the vicinity of the DSB site.

Non-homologous recombination is related to HR and occurs in mammals (but not lower eukaryotes) more frequently than homologous recombination (Roth *et al.*, 1985). This error-prone process does not depend on homology stretches and fixes DSB by end joining. Thereby arbitrary introduction of several base pairs (bp) giving rise to frame shift mutations can occur.

1.3 Site specific recombination

Site specific recombination (SSR) is a process mainly restricted to prokaryotes and lower eukaryotes like yeast but the respective machinery is also effective in higher eukaryotes like plants (maize) or *Drosophila melanogaster* (Kolb AF, 2002). SSR mediates partitioning of bacterial genomes and helps to catalyze integration and excision of bacteriophages into host DNA. It is subdivided in conservative SSR and non-conservative SSR.

Non-conservative SSR

Non-conservative SSR, also referred to as transposition, is again subdivided into the replicative model and the cut and paste model. In the replicative model a DNA fragment is copied and pasted into a new genomic location whereby each transposition increases the copy number of the transposable element. In the copy and paste model the transposable element is cut out by an enzyme called transposase. During that process, minor changes in the nucleotide sequence can occur (Sadowski, P.D., 1993). Transposition does not require sequence homologies, implying the circular transposable element can randomly integrate in adjacent positions of the genome after excision. Recently, this system could be exploited for genetic engineering purposes by expressing the transposase on a separate plasmid and

introducing a sequence of interest into the region of the transposable element on a second construct (Ivics *et al.*, 2009).

Conservative site specific recombination

For conservative site specific recombination the enzymes involved catalyze the exchange of DNA strands, which do not share obvious homologies (Stark *et al.*, 1992). They don't need exogenous energy sources like adenosine-5'-triphosphate (ATP). Unlike non-conservative SSR, here, short stretches of sequence homology (30 bp - 200 bp) are necessary for triggering recombination. In general, these recognition sites (RS) consist of two inverted sequences, which surround an asymmetrical core (spacer). These sequences (repeats) represent binding sites for the recombinases. After binding two RSs are aligned and the process of recombination is triggered. An outcome of this DNA-protein complex is a precise reciprocal exchange of DNA stretches by cut and rejoining at the asymmetrical core sequence (Kilby, N.J., 1993; ch. 1.3.3).

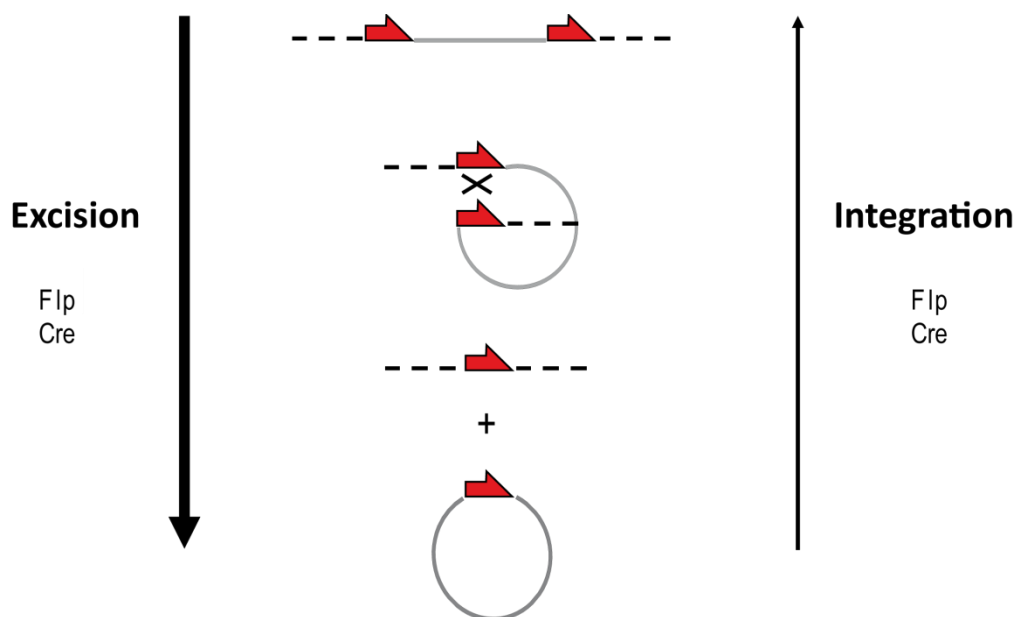


Fig 1.1. Mechanism of site specific recombination: excision and integration

If two site specific recombinase recognition sites (RS) (red half arrow) have an identical orientation, a sequence (grey string) flanked by them, will be excised (for Flp the “flip-out” reaction). The product of this intramolecular process is a circular sequence bearing one RS and a single RS left in the genome. The reversal of this reaction is the SSR-mediated integration of the circular DNA into the host DNA (called “flip in” for the Flp-process). Integration is less favored than excision, because it is a bimolecular process, which decreases the entropy (Bode *et al.*, 2000b).

In most systems the recognition sites are various for each recombinase and differ widely in their complexity but remain unchanged after recombination (Stark *et al.*, 1992). For instance, some of them need binding of accessory proteins next to the recombinase (Sadowski, P.D., 1993).

If two identically orientated RSs flank a stretch of DNA, upon application of recombinase, flanked DNA sequences are excised (Fig. 1.1). The products of this reversible process are a circular DNA sequence with one RS and the shortened genomic DNA sequence, with one RS left. Integration is the bimolecular reversal of this reaction, which lowers entropy and is much more inefficient than mononuclear excision. If both sites have a reverse orientation, the flanked DNA sequences are inverted after recombination (Fig. 1.2). While inversion and excision are intramolecular processes, integration (also called “addition”) is defined as an intermolecular reaction between two RSs on different not covalently linked DNA entities.

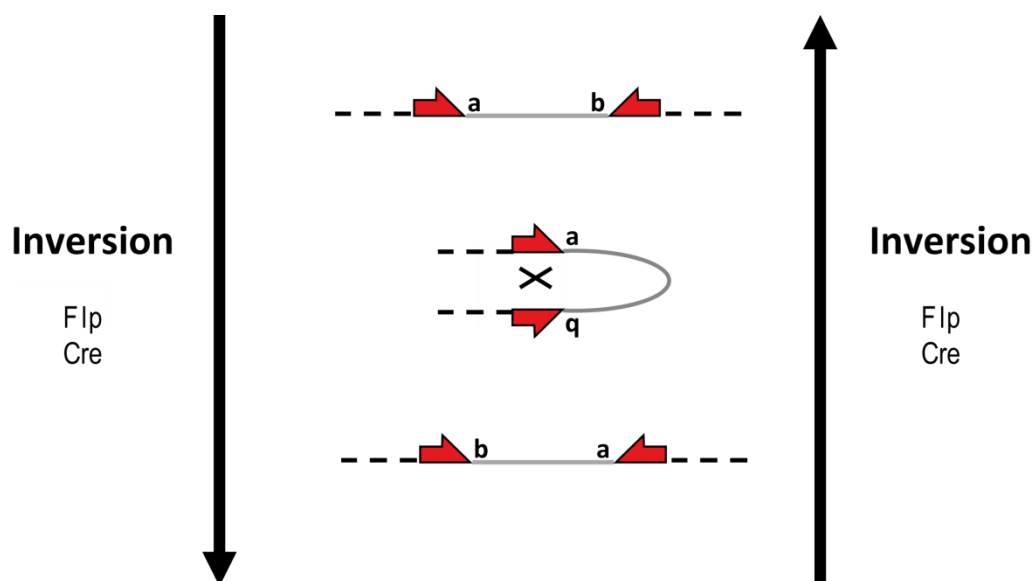


Fig. 1.2. Mechanism of site specific recombination: inversion

If a DNA stretch (marked in grey) is flanked by two inversely oriented recognition sites, SSR leads to inversion of the DNA fragment. If recombinase is present, the DNA will be continuously be inverted.

1.3.1 Serine and Tyrosine recombinases

Serine recombinases

Serine recombinases are a class of conservative SSRs, in which a serine residue is found to be critical for transient binding to DNA during strand cleavage and rejoining processes (Smith *et al.*, 2002). Serine recombinases cut four DNA backbones during recombination before they proceed with strand exchange. Thereby no Holliday junction is formed (Kolb, A.F., 2002). Resolvases catalyze the excision of a DNA sequence which is flanked by two equally oriented RSs. Invertases mediate the inversion of a DNA fragment, which is flanked by two inverted recognition sites (Sadowski, P. D., 1993). Several recombinases of this group, especially Φ C31 were shown to be feasible for genetic engineering in eukaryotes and mammalian cells (Smith *et al.*, 2002).

Tyrosine recombinases

The most prominent recombinase family is the λ integrase family, which comprises more than 100 members (Chen & Rice, 2003). Argos *et al.*, 1986 showed eight members of this family to have distinct features, for instance a well conserved homologous C-terminal region, which harbors a catalytically core consisting of arginine, histidine and tyrosine as an active triad. Tyrosine was shown to be the key player for a transient covalent linkage to the DNA during strand cleavage and rejoining. Unlike Serine recombinases, tyrosine recombinases cut one strand of each DNA molecule involved and complete this process by strand exchange. Here, similar to HR, a Holliday junction is formed. After completion of the first exchange, the second DNA strand is cut and exchanged in an analogous manner (Kolb, A.F., 2002; Fig. 1.3). The most prominent members of the integrase family are the recombination systems Cre/loxP of the bacteriophage P1 and Flp/FRT from yeast *Saccharomyces cerevisiae*.

1.3.2 Recombination system Cre/loxP

Cre (causes recombination) is a 38 kDa member of the integrase family. This tyrosine recombinase has been isolated from the bacteriophage P1 and was shown to enable maintenance of the phage in its host *Escherichia coli*. While Cre is present it splits phage dimers into monomers enabling continuous segregation of phage monomers

into daughter cells after cell division (Austin *et al.*, 1981). The 34 bp recognition site of Cre is called loxP (locus of crossover in P1). It is composed of two identical 13 bp repeat sequences, which are positioned around an 8 bp core spacer sequence (Hoess *et al.*, 1982). The repeats serve as binding sites for Cre monomers, which trigger the recombination by introducing 6 bp staggered nicks into the 8 bp loxP spacer (Hoess & Abremski, 1985). This is followed by forming of a Holliday junction visible by DNA crossovers between the spacers of the two participating lox sites. Depending on the number and orientation of loxP sites, the outcome of the recombination process might be excision, inversion or integration of loxP flanked DNA (cf. Fig. 1.1 and 1.2). The spacer of the loxP site is asymmetrical, which is necessary for determining polarity of the recognition site. Hoess *et al.*, 1986 have shown that DNA flanked by loxP sites with a symmetrical spacer is equally excised and inverted, whereas experiments with asymmetrical spacer showed excision or inversion, if Cre was present. Cre can recombine without additional supply of accessory proteins (Abremski & Hoess, 1984). Thus, it could be expanded for use in other organisms and was shown to act in mammalian cells at the transient (Sauer & Henderson, 1988b) or the stable state resulting in excision (floxing) of a transgene flanked by loxP sites (Sauer & Henderson, 1989). Lakso and colleagues (1992) could show Cre activity in mice. They crossed mice with a constitutively expressed *cre* transgene with mice harboring a transgenic oncogene that could be activated by an eye tissue-specific promoter upon Cre-mediated excision of a 1.3 kb stop sequence. All progeny mice harboring both alleles developed tissue specific eye cancers proving Cre activity in mice. Similar studies were performed by Orban *et al.*, (1992). Due to its high efficiency in mammalian cells, the Cre/loxP system has been preferentially used in embryonic stem (ES) cells and mice for excision of selection marker, which can severely impair gene expression (Pham *et al.* 1996) or the creation of conditional knockout mice (Kuehn & Schwenk, 1997, ch. 1.4.2).

1.3.3 Recombination system Flp/FRT

Saccharomyces cerevisiae contains a 2 μ m circle (6318 bp), which can replicate autonomously in its host in a process called “benign parasitism” (Volkert *et al.*, 1989).

It comprises two inverted repeats of 599 bp each (Hartley & Donelson, 1980). Both inverted repeats comprise a Flp recognition target (FRT) site, which mediates site specific recombination by pairing with another FRT site in the sibling repeat (McLeod *et al.*, 1986). The responsible enzyme for this reaction is the Flp-recombinase, which is expressed on the 2 μ m plasmid and binds to the FRT sites to promote recombination. Futcher, B., (1986) proposed a model for amplification of the 2 μ m circle to maintain copy number and segregation of the plasmid to the daughter cells. After Flp-mediated recombination one half of the 2 μ m circle is inverted. The circle is replicated by a mechanism that uses two replication forks, which both start at an origin and proceed in opposite directions. If during replication both forks are on different halves of the plasmid, Flp-mediated inversion of one half also inverts the direction of a replication fork. This situation causes both forks to run in one direction enabling continuous replication of the circle. The formed concatemers comprise several 2 μ m plasmids, which are subsequently processed by Flp-mediated or homologous recombination into monomers.

The FRT site (48 bp) consists of two inverted 13 bp repeats a and a' surrounding an 8 bp asymmetrical core (spacer) region (Hartley & Donelson, 1980) (Fig. 2.1). A third repeat b is positioned next to repeat a, interrupted by a single base pair. Non-specific nuclease digestion experiments could show that all three repeats are binding spots for Flp-monomers (Andrews *et al.*, 1985) while only the minimal FRT site including repeat a, a' and the spacer are obligatory for recombination (Amin *et al.*, 1991). The molecular mechanism of Flp-mediated site specific recombination is triggered by the binding of four Flp-monomers to the 13 bp repeats of two FRTs. The protein-DNA complex is of tetrameric nature. The N-terminus of Flp recognizes the first five base pairs (position 1-5) of the repeat, which are in direct proximity to the spacer. A direct contact is established with guanine at position 1. The C-terminus interacts with base pairs 6-10 and establishes direct contact to guanine at position 7 (Bolusani *et al.*, 2006). Two Flp-protomers located on different FRTs introduce a single cut between the thymidine (Fig. 2.1, spacer position +4/-4) and the cytosine of the adjacent repeat. Both protomers cut at the spacer of the FRT they are bound in a *trans*-horizontal way (Lee *et al.*, 1994; Lee *et al.*, 1999; Fig. 1.3). These Flp-protomers generate an 8 bp protruding overhang (Andrews *et al.*, 1985). The 3'-phosphate covalently binds to a tyrosine residue (Tyr 343) provided by a *trans*-horizontally lo-

cated Flp-protomer. Thereafter, phosphotyrosyl-bonds are cleaved to allow 3'-phosphate residues to be ligated to the open 5'-hydroxyl ends of the opposite DNA strand generating a Holliday junction. Subsequently, the cut and religation processes described above are repeated for the second DNA strands to yield final recombination products (Fig.1.3).

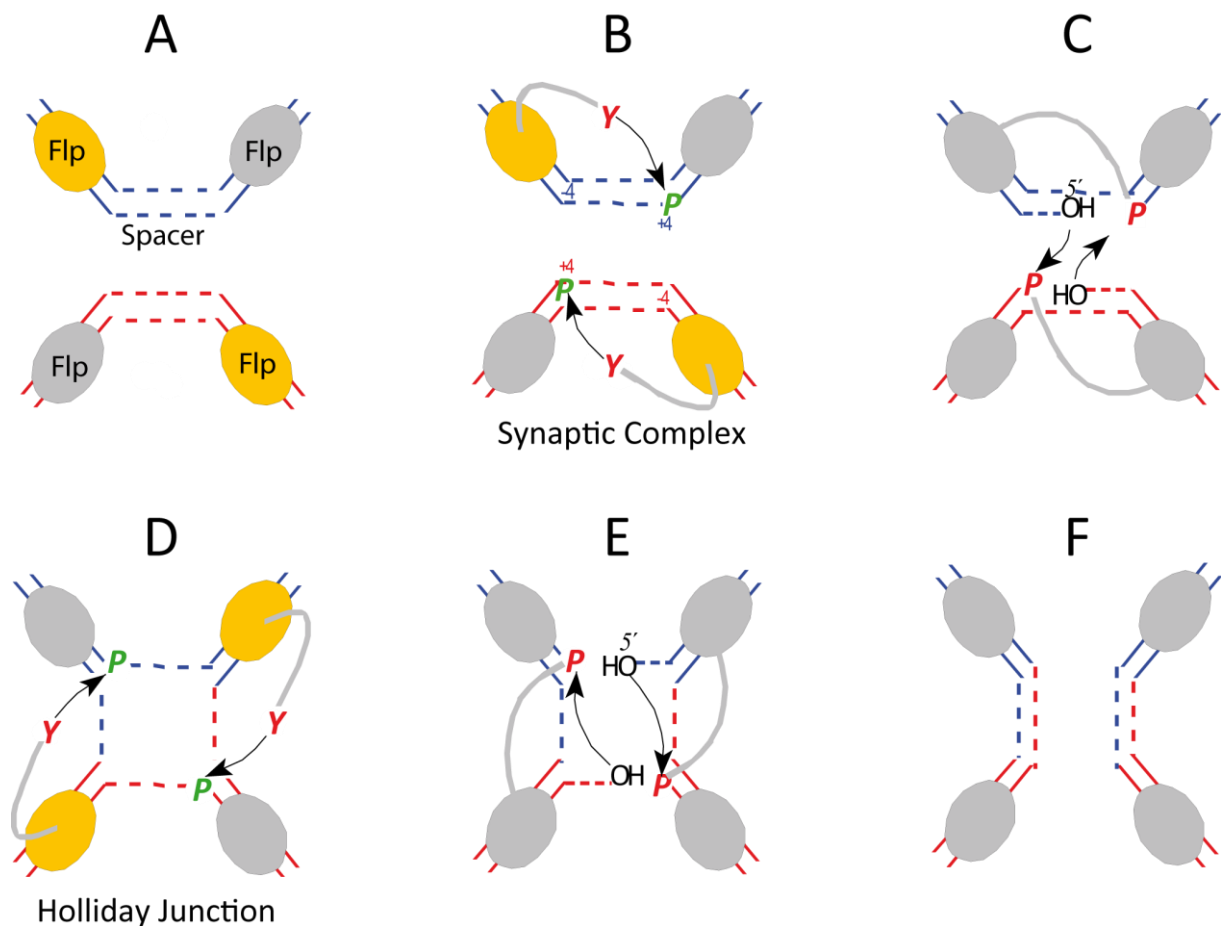


Fig. 1.3. Schematic illustration of the molecular mechanism of Flp-mediated site specific recombination of a minimal site

The starting process of Flp-mediated recombination is characterized by the alignment of two FRT Sites. Both central inverted repeats of the FRT site, which are interconnected by an 8 bp spacer, are bound by Flp-protomers (A). Two active Flp-protomers (colored in yellow) introduce an 8 bp 5'-protruding overhang at the spacer's margin (+4/-4) located at the *trans*-horizontal DNA strand (B). Both 3'-phosphate residues covalently bind to the Flp-derived tyrosine (Tyr 343) residue (depicted as Y) of the catalytic triad (B). Phosphotyrosyl-bonds are cleaved and phosphate ends rejoin with the 5'-hydroxyl-residues of the opposite DNA strands (C) to form a Holliday junction (D). After resolution of the Holliday junction (D) the analogous process (A-D) is repeated at the second DNA strand (E). After resolution of the second Holliday junction, the reaction product is formed (F).

Similar to the Cre/loxP system Flp/FRT does not need additional expressed factors like ATP or a special DNA topology and is feasible in higher eukaryotes, plants, cell lines or animals, which was illustrated by several proof of principle reports.

E. coli:

- Cox, M., (1983) showed evidence for intramolecular recombined 2 μ m circles. Jayaram, M., (1985) described intermolecular addition of two circular DNA entities.
- Flp-mediated editing of the bacterial chromosome by was shown by Huang *et al.*, (1991).

Plants:

- Flp-mediated episomal excision and integration, as well as genomic excisions in maize and rice cells were demonstrated by Lyznik *et al.*, (1993).

Drosophila melanogaster:

- Intermolecular recombination between two FRT bearing plasmids could be shown in preblastoderm *Drosophila* embryos (Konsolaki *et al.*, 1992)
- Genomic deletion of a FRT flanked (flirted) allele was previously reported (Golic *et al.*, 1989).

Mammalian cell lines:

- O’Gorman *et al.*, (1991), were the first to report the integration of a FRT bearing plasmid into a predefined FRT tagged genomic locus (“Flp-in”) which opened the possibility to target specific loci by plasmids in the genome.
- Flp-mediated excision was shown in ES-cells by deletion of genomic FRT flanked regions, which interrupted expression of a transgene. Upon recombination, transgene was expressed and could be obtained in the cells. (Jung *et al.*, 1993; Dymecki *et al.*, 1996).
- Flp-mediated elimination of selection markers in ES-cells became a widely used tool for genetic engineering (Fiering *et al.*, 1995). The stable expressing transgenic *flp*-allele in mice was sufficient to recombine genomic target sites in

a tissue specific manner *in vivo* as shown by cross-breeding experiments (Dymecki, S., 1996).

1.3.3.1 Modification of Flp

Flp-recombinase is derived from yeast and thus its optimal activity is at 30°C explaining its inferior performance in mammalian cell lines or ES cells, which grow at 37°C. 'Flp-enhanced' (Flpe) was developed in a protein evolution strategy. It showed an improved thermostability and was five-fold more active in mammalian cell culture than normal Flp (Buchholz *et al.*, 1998). Due to the reason that Cre was still more efficient than Flpe in cell culture and animal models, the codon-optimized version of Flpe 'Flp-optimized' (Flpo) was developed (Raymond & Soriano, 2007). Flpo uses the same amino acid sequence like Flpe but was shown to have recombination efficiencies similar to Cre in mammalian cell culture (Raymond & Soriano, 2007) and a five-fold higher recombination efficiency than Flpe in ES cell culture (Jae Huh *et al.*, 2010). A subsequently conducted study indicated equal performance of Flp compared to Cre recombinase in excision (Kranz *et al.*, 2010) and inversion setups.

For drug-induced activation of Flp or Cre expression estrogen receptor (ER) was fused to the C-terminus of Flp (Logie & Stewart, 1995) or Cre (Metzger *et al.*, 1995). These fusion proteins cannot enter the nucleus to mediate site specific recombination, because they are sequestered by Hsp90 to form a complex. If tamoxifen is applied, this complex is disrupted and Flp or Cre can enter the nucleus. These inducible FlpER or CreER fusion proteins are appropriate for engineering conditional knock outs (see below).

1.4 Modification of the eukaryotic genome

The modification of genomic sequences is a wide field in science. Several methods are available for altering genomic sequences. One of them is to integrate exogenous DNA, which is incorporated into the cell by transduction, transfection or electroporation. The DNA delivery can be of viral or non-viral nature. These sequences are

stably integrated into the host genome, either by illegitimate recombination or by the action of viral integrases and functions of expressed transgenes, usually under conditions of overexpression (Palmiter *et al.*, 1982). During the last decades clinical approaches like gene therapy emerged, in which the correction of mutated phenotypes was achieved by integration of correct copies of the gene mostly by viral vectors (Kay *et al.*, 2001). A remaining problem was the fact that the locus of integration is mostly random and side effects as activation of (proto)-oncogenes can occur.

1.4.1 Gene targeting by homologous recombination

With the discovery of homologous recombination in mammalian cells the targeted modification of genes came within reach. Capecchi, M., (1989) described methods for the generation of 'knock out' and 'knock in' reporter mice making use of gene targeting. Gene targeting is the alteration of genomic sequences by targeted addition of foreign DNA or deletion of endogenous DNA at a specific gene locus. For gene targeting, exogenously applied DNA is necessary. With the help of homologous recombination traceable sequences like selection markers, which are flanked by 5' and 3'-homology arms can be integrated into the genomic locus, e.g. for generation of knockout cell lines or knockout ES cells. Modified (ES) cells can be enriched by several selection methods like drug selection with Geneticin (G418) or hygromycin.

Homologous recombination is frequent in bacteria and yeast but rare in mammalian cells. This might be ascribed to the dimension and complexity of the mammalian genome. Nevertheless, the reconstruction of a functional thymidine kinase gene in murine L cells was the first report using homologous recombination for gene targeting (Lin *et al.*, 1985). Doetschman *et al.*, (1987) showed that a mutant X-linked hypoxanthine-guanine-phosphoribosyltransferase (HPRT) could be corrected by homologous recombination. This phenotype can be selected without any auxiliary elements. In order to allow the selection of genes, which are naturally non-selectable, Smithies *et al.*, (1985) modified the β -globin locus by targeted insertion of a neomycin cassette. This method inactivates the open reading frame (ORF) of endogenous genes by introduction of selection marker ('knock out') (Capecchi M.R., 1989). The

targeted 'knock out' of genes could be later successfully applied to murine ES cells. Thomas & Capecchi (1987), showed targeted disruption of a gene in murine ES cells, which serves as a common model for generation of knock out ES cells today.

1.4.2 Generation of conditional 'knock out' mice with the help of SSR

Evans *et al.*, (1981) described isolation and culture of murine embryonic stem cells. Later on, successful transplantation of genetically modified ES cells into blastocysts and generation of chimeric adult mice indicated feasibility for generation of 'knock out' mice (Bradley *et al.*, 1984). Until today, 11.000 murine 'knock out' models have been generated, 15 % of which have a lethal phenotype and cannot be dedicated to studying developmental steps as organogenesis. With the advent of site specific recombination techniques, generation of conditional knock out mutants became feasible. loxP or FRT sites are used to flox (flank by lox-sites) or flirt (flank by FRT-sites) essential exons (Gu *et al.*, 1994). Breeding of transgenic animals with conditional Cre- or Flp-expressing mice can generate conditional 'knock out' progeny harboring both transgenic alleles. These mice have a wild type phenotype until the recombinase gets expressed. Initiation of expression of the recombinase can depend on tissue specific or time/space active promoters, which allow time dependent or tissue specific knock out models (Rajewsky *et al.*, 1996). For time specific activation the drug induced activation of Flp or Cre expression by tamoxifen (ch. 1.3.2.1) or doxycyclin (Kistner *et al.*, 1996) is applied for the conditional inactivation of alleles.

1.4.3 FLEx is a genetic switch to monitor correct gene ablation in conditional 'knock out' mice

The first generation of conditional 'knock out' mice used reporters simply being flanked by loxP or FRT sites. After breeding, excision and establishment of the 'knock out' phenotype was not traceable by any reporter activity. It was shown that restricted activity and limited accessibility of the chosen recombinase could give rise to a mosaic phenotype (Nagy, 2000). Also it had to be concluded that the few transgenic lines harboring a reporter showed to be prone to silencing (Metzger & Chambon,

2001) or subject to position variegation effects (Montoliu *et al.*, 2000). This provoked faulty conclusions from data sets derived from conditional knock out models. In order to overcome these consequences, a genetic switch (FIEEx) for precise monitoring of gene ablation in conditional mice was introduced by Schnütgen *et al.* (2003). This method employs the ability of Cre to invert a floxed DNA fragment. After inversion, immediately excision of one lox site takes place, which locks the inverted DNA cassette in place. While reporter 1 (endogenous gene) is expressed in the absence of Cre, after inversion, reporter 2 (*lacZ* or *egfp*) gets activated, whereas reporter 1 is knocked out.

1.4.4 Gene Trap combined with the FIEEx-ing principle

Gene trapping was first described by Friedrich & Soriano, (1991) and is a genetic screen to introduce mutations across the murine genome and an alternative approach to generate 'knock out' models. The vectors used to this end have features in common such as a splice acceptor (SA), a promoterless reporter, which is a *lacZ*-neomycin fusion gene (*β geo*) and a sequence coding for a poly adenylation tail (poly(A)) in 5'-position of the reporter sequence. After random insertion of a lentiviral vector into an intronic region of a gene (usually the first intron), the endogenous promoter can activate expression of the reporter, which may be spliced, via the co-introduced SA, to the exon(s) located downstream. In early approaches, however, the expression of the endogenous fusion transcript is abrogated by the expression of the poly(A), which resides downstream to the reporter. Chimeric gene trap mice allow tracking of expression of the reporter *in vivo* and give rise to gene functions. As described before (ch. 1.4.1) common gene traps are not appropriate for genes, which cause lethality by 'knock out'. Therefore, gene trap and FIEEx had to be combined to generate conditional retroviral gene trap vectors, which can be regulated by expression of site specific recombinases as Cre or Flp (Schnütgen *et al.*, 2006). With the help of this technique 7013 genes could be assembled in an ES-library (Schebelle *et al.*, 2010).

1.5 Recombinase-mediated cassette exchange (RMCE)

As mentioned above, homologous recombination and gene trapping are widespread tools to generate ‘knock out’ models. These techniques are hampered by the necessity of selection marker, which can induce silencing (Artelt *et al.*, 1991, Kim *et al.*, 1992) or unpredictable effects on gene expression of neighboring genes (Pham *et al.*, 1996, Fiering *et al.*, 1995). Several reports used Cre or Flp to delete selection markers after targeted integration.

Askew *et al.*, (1993) and Wu *et al.*, (1994) described the ‘tag and exchange’ operation, which combines ‘knock out’ with ‘knock in’. After a HR-mediated ‘knock out’ of a gene, accompanied by integration of a selection marker, the locus can be retargeted subsequently in order to replace the selection marker by a mutated wild type sequence of the initially knocked out gene. This method was developed to characterize the role of specific amino acids of the desired gene *in vivo*. Nevertheless, the low frequency of homologous recombination, which ranges between 10^{-5} and 10^{-7} of transfected cells (Yanez & Porter, 1998), make this approach very laborious. Significantly higher frequencies of retargeting a locus could be achieved with recombinase-mediated cassette exchange (RMCE).

Schlake & Bode (1994) developed this technique by introduction of a pair of hetero-specific 48 bp FRT sites F_{wt} and mutant site F_3 . F_3 was obtained by mutating 4 positions of the spacer region, which still maintains a full self-recognition potential to identical partner sites as anticipated by studies on series of mutant FRTs (Umlauf & Cox, 1988). Both FRT sites are able to recognize identical (homospecific) partners ($F_{wt} \times F_{wt}$, $F_3 \times F_3$) but not the different (heterospecific) counterparts. Schlake & Bode, (1994), concluded that a genomically integrated selection marker, which is flanked by heterospecific FRTs F_{wt}/F_3 can be swapped by an exogenously applied supercoiled exchange vector, which harbors a gene of interest (GOI) but is flanked by the identical set of FRTs (modified “Flp-in” principle). In detail, this Flp-driven reaction is suited to replace the parental selection marker (usually a positive-negative selection marker) by successive addition and excision steps with regard to the donor DNA sequence and the parental sequence. The excess of transiently available donor plasmids drives the kinetically unfavored bimolecular reaction. During this process, removal of selection markers, being present in the unmodified locus, prevent sub-

sequent transcriptional silencing effects to occur. For instance, with the help of negative selection marker thymidine kinase (tk), which is removed after exchange, targeted events can be augmented by the application of Ganciclovir (Fig. 1.4)

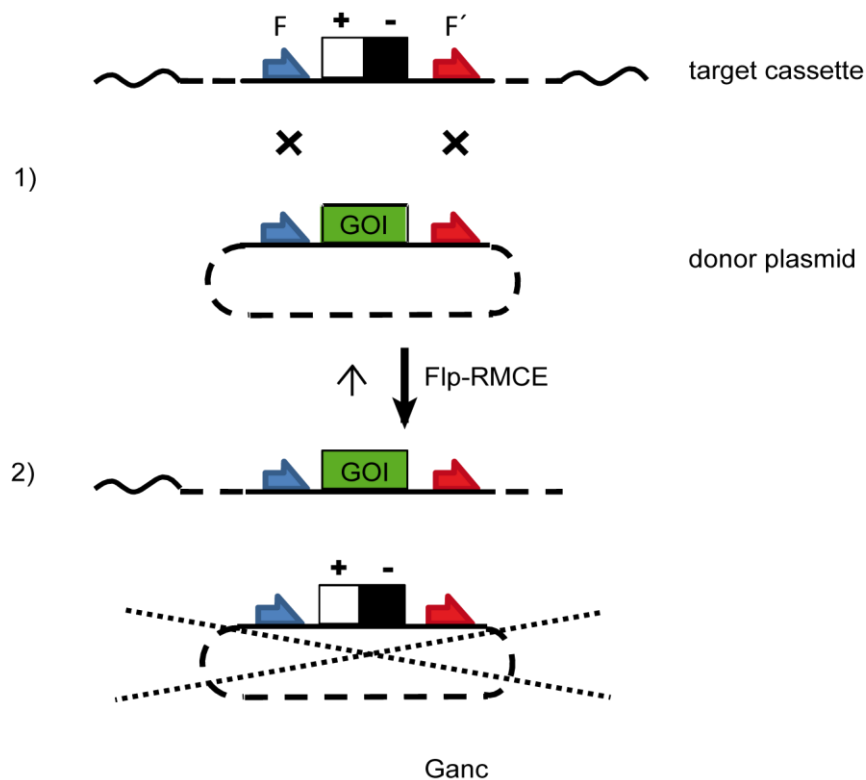


Fig 1.4 Schematic sketch of Flp-recombinase-mediated cassette exchange (RMCE)

A genomically integrated positive/negative selection marker, for instance hygromycin/thymidine kinase (hyg^{tk}) is flanked by a set of heterospecific FRT sites (F-F'). RMCE is triggered by co-transfection of a Flp-encoding plasmid and a donor, which bears a F-F' flanked gene of interest (GOI) (1). Site specific recombination of both sets of FRTs leads to the exchange of the FRT flanked region of the target by the donor. One product of this reversible reaction is the genomically integrated GOI at the original locus. The excised positive/negative selection marker forms an episome after RMCE, which gets dismantled by the host cell (2). Ganciclovir permits the enrichment of cells for which RMCE has taken place. An excess of the donor plasmid is suitable to drive the reaction into the desired reaction (integration). Note that the prokaryotic plasmid parts of the donor (dashed line) are not co-introduced, which makes RMCE superior to most similar targeting approaches.

In contrast to the approach by O'Gorman, (1991), no prokaryotic vector parts, which can cause silencing (Riu *et al.*, 2005) and were reported to severely impair expression kinetics of adjacent genes (Townes *et al.* 1985) are left behind. Silencing can be triggered by DNA-methylation of a cytosine, which is part of a cytosine-

guanine (CpG)-dinucleotide (Kass *et al.*, 1997). Due to the fact that prokaryotic sequences harbor significantly more CpG-dinucleotides than eukaryotic sequences it was concluded that prokaryotic sequences, stably integrated in eukaryotic genomes, are more prone for silencing effects.

After its first description by Schlake and Bode, (1994) RMCE was applied for several cell lines (Goetze *et al.* 2005,) and murine ES cells (Schübeler *et al.* 1998, Lauth *et al.* 2002, Cobellis *et al.* 2005). Qiao *et al.*, (2009) reported generation of high expressing clones for pharmaceutical protein expression systems. In this strategy, the unique benefit to retarget a precharacterized genomic locus by RMCE was exploited and technically refined. Cesari and colleagues, (2004) applied RMCE for generating *elk-1* murine 'knock out' ES cells and chimeras. With the help of RMCE three different mutant *lrp* ES-lines and chimeric mice were created. These studies showed the superiority of RMCE, in contrast to HR, for the time saving construction of several mutant ES cell lines. In comparison to tag and exchange methods, which made use of two successive homologous recombination steps (Askew *et al.*, 1993; Wu *et al.*, 1994) RMCE proved to be more straight forward and much more efficient (Roebroek *et al.* 2006). Recently, the feasibility of retroviral FIEEx gene traps for RMCE was described by Schebelle *et al.*, (2010). Here, a murine ES cell library of 85.742 annotated loci was established, all of which bear a set of heterospecific FRTs, which can be subjected to RMCE, to insert any given cDNA sequence to a genetic target of choice.

1.5.1 Cre- and Φ C31-RMCE

Hoess *et al.*, (1986) created the heterospecific loxP variant lox511, which could be successfully used for the elaboration of Cre-RMCE in NIH/3T3 cells (Bethke & Sauer 1997). Further reports showed Cre-RMCE in ES cells (Feng *et al.*, 1999). Nevertheless, several reports claimed cross-recombination among heterospecific loxP sites (Kolb *et al.*, 2001; Langer *et al.*, 2002) and occurrence of genomic lox pseudo sites (Schmidt *et al.*, 2000), which are severe setbacks for the further development of Cre-RMCE (ch. 3.5).

RMCE with the serine recombinase Φ C31 was shown to be functional in *Drosophila* (Bateman *et al.*, 2006) but both recognition targets are not appropriate for iterative use of RMCE. Genomic pseudo sites have been traced for this and other recombinases restricting the use of Φ C31-RMCE to single targeting steps (Oumard *et al.*, 2006).

1.6 Multiplexing-RMCE

The first proposal for using multiple (natural) loci of loxP pseudo site variants in yeast for the targeted integration of loxP-tagged vector by Cre goes back to Sauer, (1996). Later, studies proposed Flp-RMCE for multiple targeting of cell lines. (Bode *et al.*, 2000b).

In contrast, to address a single locus by RMCE, multiplexing uses different sets of heterospecific FRTs as genomic addresses, each of which can be targeted by a specific donor vector. The difference among each combination of 'parental tag' and 'donor vector' is the composition of the flanking FRTs. Both FRT sets must not constitute recombination partners for each other, in order to prevent mistargeting events or unwanted chromosomal rearrangements. Each parental vector bears a different positive selection marker, for instance neomycin in cassette 1 and hygromycin in cassette 2 for initial drug selection of doubly tagged cells. After exchange of both cassettes, FACS sorting of double positive fluorescent markers and negative selection with Ganciclovir can enrich doubly targeted cells (Fig. 1.5)

Other obstacles had to be overcome to make the approach useful: The originally proposed pair F₅-F₃ (Bode *et al.*, 2000b) had to be abandoned, because F₅ and F₃ showed evidence for cross-recombination in transient pioneer studies (Schlake & Bode 1994; see below). Owing to this fact, novel heterospecific FRTs had to be designed and tested for functionality also regarding unwanted, promiscuous recombination events between the loci (Turan, S., diploma thesis, TU-Braunschweig, 2007).

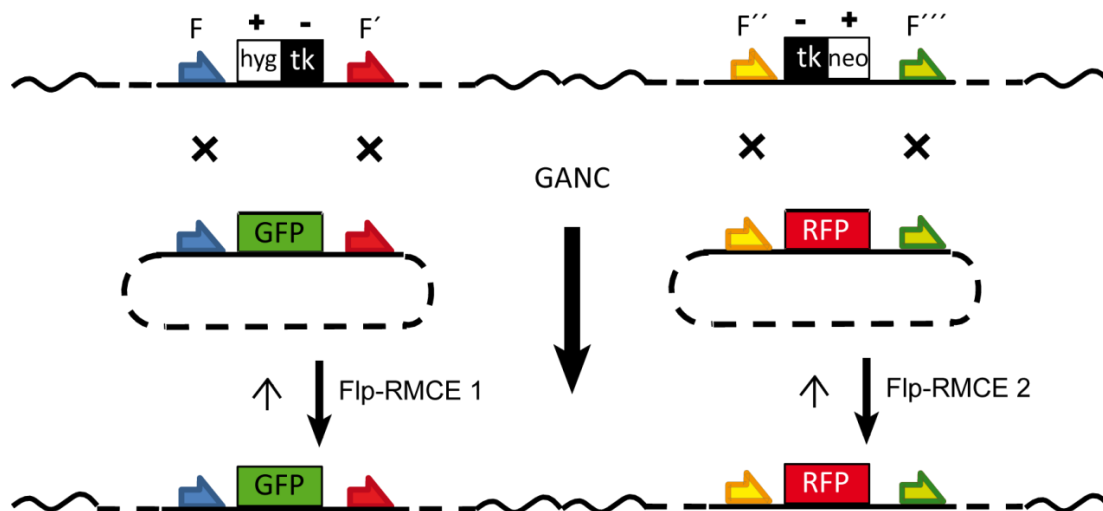


Fig. 1.5 Multiplexing RMCE

The genomic integration of two parental cassettes each provided with a different set of heterospecific FRTs (F-F' or F''-F''') allows multiplexing RMCE. Transfection of two donor molecules, which were provided with appropriate set of FRTs and a Flp-expression vector allows simultaneous RMCE at two distinct loci. This project is based on the exchange of two selection marker (hyg^rtk and tk^rneo) by GFP or RFP, respectively. Doubly exchanged cells can be enriched by FACS sorting and negative selection via the tk-GANC system.

1.7 Aims of the project

Primary aim of this project was to show a proof of principle for Multiplexing-RMCE in a mammalian cell line. The verification of multiplexing-RMCE depends on creation and testing functional FRT mutants. A first approach was the construction of seven novel FRTs according to rules, which determined parameters for self-recognition (Umlauf & Cox, 1988) and cross-recombination (Schlake & Bode 1994). The first PCR test series examined recognition and cross-recombination potential of novel FRTs and were done in a previous study (Turan, S., diploma thesis, TU-Braunschweig, 2007). Results provided first indications for effective mutants regarding both, function and safety. While PCR is suitable as a straightforward pre-test to elucidate self-recognition and cross-recombination potentials of single FRTs, it does not provide sufficient data, to derive optimal sets of FRT sites in an RMCE context. To this end a more dedicated test (transient RMCE, as introduced by Seibler *et al.*, (1997) was applied to demonstrate, which combination of heterospecific FRTs is optimally suited for a multiplexing-RMCE setup in a genomic environment. Besides

the conventional combinations (F_3 - F_{wt} and F_5 - F_w) several novel combinations should be examined for exchange efficiency and safety.

At least two pairs of FRT site should be identified and implemented into parental vectors and donors. Parental vectors should be stably integrated into the NIH/3T3 cell clone N1. The N1 clone harbors one extensively characterized genomic locus carrying the F_3 -hyg^{tk}- F_{wt} address (Goetze *et al.*, 2005) and should be provided with a secondary target to generate multiplexing-RMCE competent cell lines. These cell lines should be characterized in terms of targeting efficiency, expression pattern of integrated transgenes and frequency of random integration events of donor vectors.

2 Results

2.1 Pre-test (PCR)

The FRT wild type (wt) site of 48 bp is composed of three repeats (a, a' and b) with a length of 13 bp each. Two repeats, inversely orientated (a and a'), flank an asymmetrical 8 bp spacer region. The third repeat b is a duplicate of repeat a' in 5'-position to this one. a' and b are separated by one base pair. The minimal functional FRT site is 34 bp long and solely formed by a' and a surrounding the spacer region (Fig. 2.1).

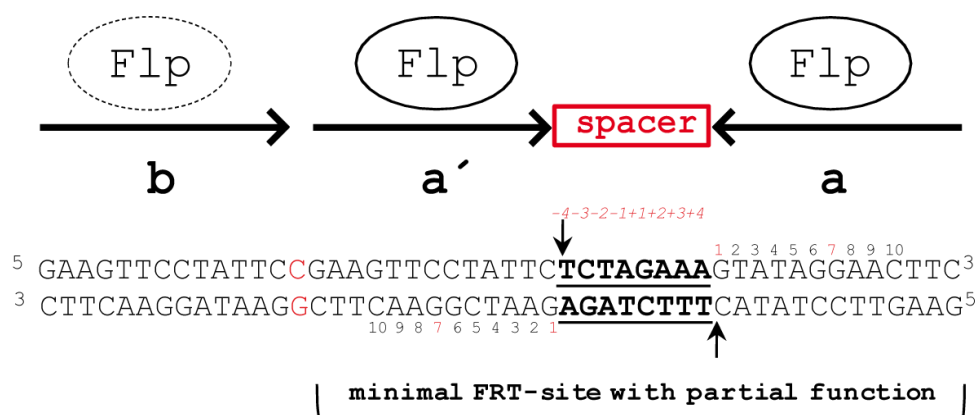


Fig 2.1 architecture of a wild type (wt) FRT site

The FRT site consists of three 13 bp repeats depicted as black arrows (a, a' and b). Repeat b and a' are disconnected by one base pair shown in red. Each repeat forms a binding site for a Flp-monomer. An asymmetrical spacer, which resides between a' and a defines orientation of the site and is the place of recombination. Brackets show the minimal, partly functional, FRT-site of 34 bp, that consists of a', spacer and a.

In extended mutagenesis studies of the minimal 34 bp FRT site Umlauf & Cox (1988) concluded that a single mutation of the central 6 bp of the spacer does not affect recombination potential if the partner FRT site possessed the identical mutation. Nevertheless, several mutations decreased recombination potential when 5'-polypyrimidine tracts reaching from the adjacent repeats to the core of the spacer were interrupted.

They could also show that mutations of the spacers' external base pairs severely reduced self-recognition potential of the FRTs. This is due to the fact that Flp-monomers have contact with the bordering base pair of the spacer (Bruckner *et al.*, 1986) and its mutation decreases binding potential of Flp. A third fact determined impaired recognition of mutants when AT-content of the spacer sequence fell below a value of 75 %. These conclusions were summarized in three rules for the creation of functional minimal FRT mutants (Tab. 2.1).

Tab. 2.1 rules obtained by Umlauf and Cox (1988) for the generation of functional new mutant FRTs shown on the right for the F_{wt} site, which serves as a reference.

	Rules	Sequence and AT-content (%) of the 8 bp spacer
Rule 1	No major interruptions of the 5'-polypyrimidine-tracts	5' TCT AGAAA 3' (75 %) 3' AGAT CTT T 5'
Rule 2	No mutation of bordering base pairs of spacer	5' TCT AGAAA 3' (75 %) 3' AGATCTT T 5'
Rule 3	AT-content of the spacer ≥75 %	5' TCTAGAAA 3' (75 %) 3' AGATCTTT 5'

They were considered in mutagenesis studies for the assembly of novel FRTs (Turan, S., diploma thesis, TU-Braunschweig, 2007). Minimal FRT sites of 34 bp were reported to be capable of intramolecular excision mechanisms but not intermolecular integration (Jayaram, M., 1985 and Lyznik *et al.*, 1996). For cassette exchange, integration is an indispensable step. Thus the architecture for the novel FRTs had to be based on the three repeat model of 48 bp size.

2.1.1 Construction of novel mutant FRTs

The pre-studies (Turan, S., diploma thesis, TU-Braunschweig, 2007) of this work included the critical evaluation of these rules with the new established FRTs. Some of these mutants were constructed following most of the previously described rules. As a negative control two mutants were developed challenging the rules (Tab. 2.2).

Tab. 2.2 The number of differences within the 8 bp spacer for each combination of sites is listed on the left part of the table. A color code marks wild type FRT site (grey), mutant FRTs, which were applied for RMCE in earlier studies (yellow) (Schlake & Bode, 1994), novel mutants constructed following rules (Tab. 2.1) (green) and violating rules (red). The spacer sequences are pictured on the right. Violations of rule 1 are shown in blue, for rule 2 in red and for rule 3 in orange. The AT-content is depicted as an elevated number on the right in percentage.

	F_{WT}	F_3	F_5	F_{11}	F_{12}	F_{13}	F_{14}	F_{15}	F_{10}	F_{16}	FRT Sites
F_{WT}	-	4	4	4	4	4	4	6	(2)	4	TCTAGAAA ⁷⁵ AGATCTTT
F_3	4	-	(2)	5	5	5	5	4	6	5	TTCAAATA ⁸⁸ AAGTTTAT
F_5	4	(2)	-	5	5	5	5	5	6	6	TTCAAAG ⁷⁵ AAGTTTTC
F_{11}	4	5	5	-	5	(3)	5	5	6	6	TGAATAA ⁷⁵ ACTTGATT
F_{12}	4	5	5	5	-	5	(2)	4	6	5	TTCTGAA ⁷⁵ AAAGACTT
F_{13}	4	5	5	(3)	5	-	4	(3)	6	5	TCATATAA ⁸⁸ AGTATATT
F_{14}	4	5	5	5	(2)	4	-	4	6	5	TATCAGAA ⁷⁵ ATAGTCTT
F_{15}	6	4	5	5	4	(3)	4	-	8	6	TTATAGGA ⁷⁵ AATATCCT
F_{10}	(2)	6	5	6	6	6	6	8	-	6	ACTAGAAT ⁷⁵ TGATCTTA
F_{16}	4	5	6	6	5	5	5	6	6	-	TCCGGGCA ²⁵ AGGCCCGT

Cassette exchange is only feasible if both FRT sites have only specific interactions with their homologous partner FRTs. Cross-interactions among FRTs that lie in *cis*-position on the same DNA strand must not occur. In order to avoid this reaction the heterospecificity between those FRTs has to be above a critical threshold.

In former studies by Schlake & Bode (1994) mutant site F_3 could be shown to fully recognize identical mutants but not the F_{wt} site. Comparing both sites' spacer, they differed in 4 bp suggesting that this difference between the spacers is sufficient for complete inhibition of cross-recombinational activity. Furthermore, the combination F_3 - F_{wt} was successfully applied for cassette exchange. In summary: to gain a new set of

FRTs feasible for (multiplexing)-RMCE three main criteria had to be addressed carefully.

- All rules unraveled by Umlauf & Cox, (1988) had to be applied mostly (Tab. 2.1).
- Each spacer of a given FRT had to differ in at least 4 positions to its partner site
- In order to work out a second cassette next to the established one (F_3 - F_{wt}) for multiplexing-RMCE studies all candidate FRTs' spacer had to differ in 4 bp compared to both F_3 and F_{wt} .

2.1.2 Characterisation of novel mutant FRTs

Seven new mutant sites were evaluated for two criteria. Due to the fact that RMCE takes place with low efficiencies, weak self recombination potential, would extremely diminish action of RMCE. Therefore the pre-test serves as a good quality control for assessment of self-recognition potential (Fig. 2.2).

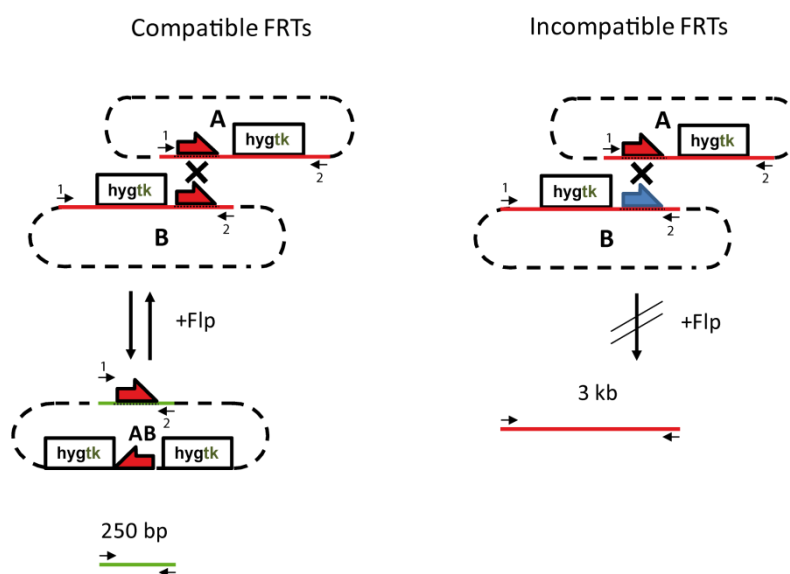


Fig 2.2 Schematic view of the pre-test

Comparison of FRT sites: construct A bearing a FRT (half arrow) in 5'-position of a hyg^rk gene and construct B, bearing a FRT in 3'-position of a hyg^rk gene are transferred into BHK-A cells. Upon recombination of homospecific (compatible) FRTs mediated by Flp-recombinase both constructs fuse to yield episomal entity AB. During that process rearrangements of primer sequences (shown as arrows, 1 or 2) take place giving rise to a 250 bp PCR-derived product. Non-recombination of heterospecific (incompatible) FRTs leads to no merge of given constructs. This situation is revealed by a 3 kb PCR-amplified fragment.

Secondly, promiscuous recombination among heterospecific FRTs in a genomic background would give rise to rearrangements due to deletions or additions. Transiently present donor plasmids could also be subject for merge reactions. With the PCR-test first evaluations of the novel mutant FRTs exemplified in Fig. 2.3 were conducted. In a first series of experiments constructs A and B harboring identical FRTs were compared with each other. Flp expression was assured with addition of expression vector Flpe-puro. For controls it was replaced by neutral DNA pBMC1.

Site specific recombination among both vectors' FRTs gives rise to a merged plasmid designated AB. Rearrangements of priming regions after recombination can be monitored via PCR analysis. If recombination takes place a 250 bp amplicon can be obtained, whereas lack of recombination leads to non-interaction between FRTs of A and B. In that case solely a 3 kb PCR product is revealed (Fig. 2.2). All seven new FRT mutants plus reference sites F_{wt} , F_3 and F_5 showed up intense bands of 250 bp size if Flpe-puro was cotransfected. A dominant band of 3 kb was found in samples in which no Flpe-recombinase was applied. The evidence for fused plasmid AB via PCR in samples transfected with Flpe-puro (Flp^+) suggests site specific recombination among homospecific pairs of seven new mutants and three conventional FRTs for this experiment. Three FRTs (F_{11} , F_{12} and F_{16}), however, showed up a decreased self-recognition potential (Fig. 2.3).

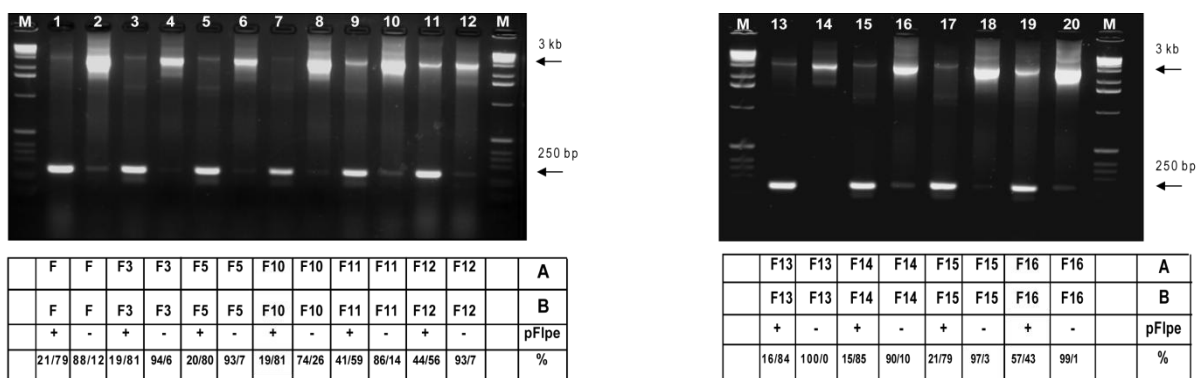


Fig 2.3 comparison of homologous FRTs in BHK-A cells

PCR products were derived as described in Fig. 2.2. First two lines of the table beneath PCR picture depict chosen FRT mutant on construct A or B. Third line indicates whether the Flpe expressing construct was applied. Fourth line shows outcome of densitometric analysis shown as a 3kb band/ 250 bp band ratio. All compared FRT sites show self-recognition potential, when Flpe is expressed indicated by an intense band of 250 bp in the Flp^+ -samples. Samples, which were transfected with neutral DNA (Flp^-), show distinct appearance of a 3kb band suggesting presence of educt vectors. M: 1kb DNA ladder.

Violation of rule 1 does not hamper effect on the recombination potential of mutants F_{10} , F_{13} and F_{15} , whereas F_{11} seems to be impaired. PCR analysis of F_{11} shows a significant band suggesting recombination along with a 3 kb band indicating no re-combinatorial action. Densitometric analyses comparing 3 kb band/ 250 bp band ratio are 41/59, whereas recombination potential of F_{wt} is reflected by a ratio of 21/79 shifted towards the 250 bp band. The impaired recognition of the F_{11} mutants might be due to the occurrence of a guanine in position -3 or the T $\leftrightarrow$ A conversion at position -2 in the spacer. The significant decrease of self-recognition for F_{12} , revealing a ratio of 44/56 cannot be illuminated by now, because the FRT was constructed following all rules for generation of mutant FRT sites. Impaired potential for self-recognition of F_{16} , which shows the highest interference of recognition (57/43) might be ascribed to the low AT-content (25 %) of this sites' spacer.

As described above, cross-recombination between heterospecific FRTs would severely affect performance of RMCE and rather lead to excisions and deletions of FRT-flanked constructs. For further investigation of this point all FRT mutants were compared to the F_{wt} site. As depicted in Fig. 2.4 none of the mutant FRTs showed any distinct recognition potential when aligned with the F_{wt} site.

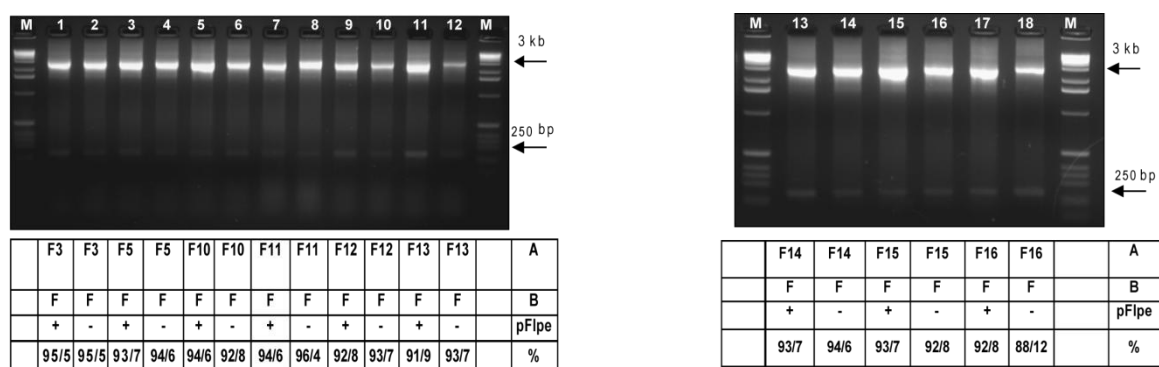


Fig 2.4 Comparison of heterospecific FRTs in BHK-A cells

All mutants (construct A, first line) were exposed to F_{wt} (construct B, second line) with or without co-delivery of the Flpe expressing construct (+/-, third line) in BHK-A cells. In all compared samples a 3 kb band is prominent, whereas 250 bp band is not detectable, which suggests no Flp-mediated recombination of educt vectors A and B. Results are quantitated with densitometric analysis of software Imagequant™. Faint 250 bp-sized band is rather due to homologous recombination than to site specific recombination.

Faint 250 bp bands arose in the Flp⁺- and Flp⁻-samples, which is also reflected by a 3 kb band/ 250 bp band ratio of approximately (88/12 - 96/4). These bands cannot be accounted by Flp-recombination, because they also appear in the Flp⁻-samples and thus might be rather due to homologous recombination among the homology stretches of plasmids A and B (Schlake *et al.*, 1994).

2.2 Transient RMCE

Pre-tests served to evaluate the functionality of newly synthesized FRTs in terms of self-recognition and inhibition of cross-recombination. Transient RMCE should serve as a more sophisticated control to clarify previously exhibited results. The aim of transient RMCE assays was to show functionality and safety of novel FRT pairs in terms of cassette exchange. Combinations of FRTs, which complied with the criteria concerning RMCE (ch. 2.1) were used for the construction of a two-vector system (promoter-outside/promoterless). For triggering transient RMCE both vectors and Flp recombinase had to be present in the cell.

Questions concerning transient cassette exchange have been evaluated in a former study by Seibler and colleagues, (1997). The authors observed that the frequency of exchange events was dependent on the chosen cell line. The immortal embryonic murine fibroblast cell line NIH/3T3 turned out to be a perfect system for RMCE. For this reason, this cell line was used for the following studies.

2.2.1 Vector architecture for Transient RMCE

The system of transient RMCE comprises two vector types “P_{rfp}” and “ΔP_{gfp}“, which are provided with the fluorescence marker gene RFPexpress (RFPex) or enhanced GFP (eGFP). These markers are flanked by two heterospecific FRT sites. The combination of marker and flanking FRTs is herein after referred to as exchange cassette (Fig. 2.5).

The orientation of a FRT site is defined by the spacer sequence (Schlake *et al.*, 1994). The position and orientation of the FRT pairs is identical in both vectors. P_{rfp}

contains a SV40 enhancer/promoter located in 5'-position outside of the exchange cassette, whereas ΔP_{gfp} bears at the identical position no promoter but an inversely oriented late SV40 poly(A) signal to reduce background expression originating from the plasmids' bacterial promoter.

After cotransfection of both vectors together with a Flp-expressing vector, a Flp-mediated double reciprocal crossingover between the homologous FRTs of both exchange cassettes can lead to the substitution of the FRT flanked markers (Fig. 2.5; cf. Schlake *et al.*, 1994). A consequence of this substitution is the activation of eGFP expression by the SV40 promoter/enhancer. One product of the transient RMCE is a novel recombined plasmid called P_{gfp} (Fig. 2.5).

In this approach five variants of P_{rfp} and ΔP_{gfp} were constructed and tested for transient RMCE. Previously characterized FRT pairs F_3-F_{wt} and F_5-F_{wt} were used as reference controls and three remaining pairs were composed of novel FRTs. FRT sites chosen to form a pair, had to differ in at least four positions of their spacer sequence. For further use in multiplexing-RMCE experiments a difference of 4 bp in the spacer region of all four used FRTs had to be given. Combinations, which complied with the major criteria, were $F_{11}-F_{12}$, $F_{13}-F_{14}$ and $F_{14}-F_{15}$. The first test concerned the (quantitative) detection of episomal exchange events when Flp was coexpressed with both provided donors. After transient RMCE the rise of eGFP expression originating from product vector P_{gfp} could be monitored by cytometric FACS analysis. In order to detect background expression of ΔP_{gfp} Flp-coding vector was replaced by empty DNA, which was merely expressing puromycin.

An additional test concerned the question if heterospecific FRT sets of two donor plasmids are prone to promiscuous cross-interactions among each other. This control was of relevance for later multiplexing-RMCE experiments, which also dealt with several colocalizing vectors. Donors with heterospecific (incompatible) sets of FRTs were cotransfected with Flp. ΔP_{gfp} derivatives for combinations $F_{11}-F_{12}$, $F_{13}-F_{14}$, $F_{14}-F_{15}$, and F_5-F_{wt} were compared with plasmid P_{rfp} , which bore the reference combination F_3-F_{wt} . Configuration $F_{wt}-F_3$ on ΔP_{gfp} was matched to $F_{11}-F_{12}$ on P_{rfp} .

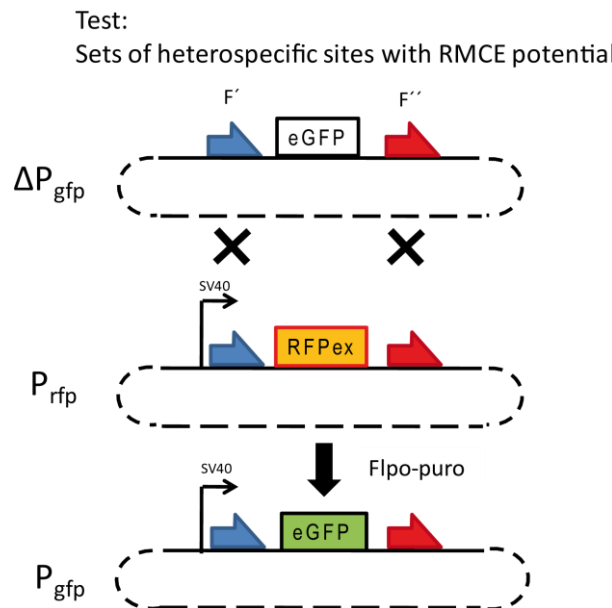


Fig. 2.5 Transient RMCE: schematic view

Depicted is ΔP_{gfp} , a silent vector carrying a promoterless *egfp* cassette flanked by a heterospecific set of FRT sites (F' and F''). P_{rfp} contains a *rfpex* gene flanked by heterospecific FRTs in identical orientation and constitution to those in ΔP . *rfpex* is under control of a constitutively active SV40 promoter/enhancer, which resides outside of the exchange cassette in 5'-position. Upon transfection of P_{rfp} and ΔP_{gfp} both plasmids are recombined at their homospecific FRT sites if Flp-recombinase (Flpo) is present. One product of this RMCE is P_{gfp} , whose *eGFP* expression is activated by the SV40 promoter/enhancer. The other product ΔP_{rfp} is not depicted.

2.2.2 Transient RMCE of novel mutant FRT pairs

24 hours prior transfection 1.5E5 cells were seeded per 6 well plate. Four hours previous to transfection culture medium was refreshed. The first series of experiments were conducted with transfection of P_{rfp} and ΔP_{gfp} , both bearing a set of FRTs and the codon-optimized version of *flpe* (*flpo*) (Raymond *et al.*, 2007).

All three plasmids were cotransfected, via lipofection, using reagent Metafectene[®] at ratio of 3:1:1. In detail, for P_{rfp} 2.4 μ g of plasmid DNA was used, for ΔP_{gfp} and Flpo-puro 0.8 μ g were transfected. For Flp⁻-controls, Flpo-expressing plasmid was replaced by neutral vector BSpac- ΔP , which drives expression of selection marker puromycin. 4 hours post transfection culture medium was exchanged by puromycin containing medium ($c = 2.5 \mu$ g/ μ l). The puromycin resistance gene is under control of the internal ribosomal entry site (IRES) element. Here, puromycin selection is sup-

posed to enrich cells, which are resistant to the selection drug due to uptake of the plasmid in the transient phase. 48 hours post transfection cells were washed with PBS and harvested via trypsinization. Expression of fluorescent markers was determined by flow cytometric analyses at the FACSCalibur™. X-axis shows eGFP-expressing, Y-axis RFPex-expressing cells on a logarithmic scale. The relative profile of eGFP/RFPex coexpressers is shown with and without concurrent Flp-activity in a FACS dot plot (Fig. 2.6 a, b). Here, Flp⁺-samples show a population of 8.9 % eGFP/RFPex coexpressers, whereas just 0.9 % of the Flp⁻-controls show a double fluorescent expression profile (cf. Fig. 2.6a, b).

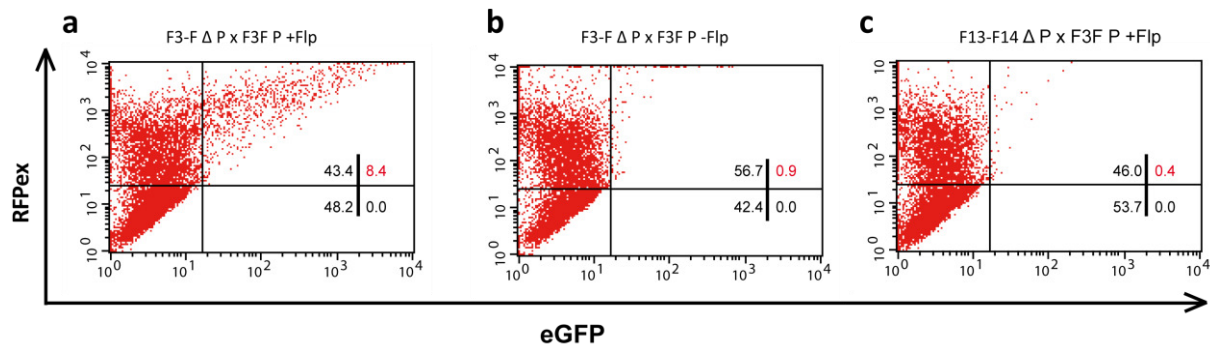


Fig. 2.6 Transient RMCE exemplified by FACS

Promoterless ΔP_{gfp} contains a silent *egfp* (G) cassette and is flanked by FRT pair F_3 - F_{wt} (F' -G- F''). P_{rfp} harbors a F_3 - F_{wt} flanked *rfpex* (R), which is under control of SV40 promoter/enhancer (SV40- F' -R- F''). These constructs were cotransfected into the NIH/3T3 cell line with (a) or without (b) Flpo (CAGGS-flpo-IRES-puro) expression vector. 24 hours post transfection culture medium was exchanged by medium containing selective agent puromycin (2.5 μ g/ μ L). 48 hours post transfection cells were collected via trypsinization and measured in a FACS cytometer. RFPex expression is detected on a logarithmic scale (Y-Axis), eGFP expression is depicted on X-Axis of the Dot Plot. **a:** Both exchange vectors P_{rfp} and ΔP_{gfp} contain an identical set of FRTs (F_3 - F_{wt}). After cotransfection of these with Flpo-puro transient RMCE gives rise to a pool of eGFP/RFPex positive cells (8.4 %), pictured in the upper right quadrant. **b:** cotransfection of vectors containing identical sets of FRTs, in which Flpo-encoding plasmid was replaced by empty-DNA, gives rise to no significant double positive cell population (0.9 %). **c:** cotransfection of vectors containing incompatible sets of FRTs (F_{13} - F_{14} and F_3 - F_{wt}) with Flpo expressing plasmid leads to no raise of eGFP/RFPex coexpressors.

The increased expression of eGFP in the samples cotransfected with Flpo-puro suggests the presence of vector P_{gfp} , which was likely due to Flp-mediated site specific recombination among the homologous FRT sets F_{wt} and F_3 of educts P_{rfp} and ΔP_{gfp} . A reason of striking occurrence of eGFP positive cells might be due to the ratio

of plasmids used for transfections. In order to shift the RMCE-ratio towards product P_{gfp} the three-fold higher concentration of educt P_{rfp} than second educt ΔP_{gfp} was applied.

To evaluate the potential of cross-recombination incidents among vectors with incompatible FRT, pairs with active Flp P_{rfp} bearing the F_{13} - F_{14} set was cotransfected with a F_3 - F_{wt} type ΔP_{gfp} plasmid plus Flpo-puro (Fig. 2.6c). Similar to Flp⁻-controls no raise of an eGFP/RFPex coexpressing population is visible here. This suggests negligible minimal cross-recombination between described FRTs. Notably, a threshold eGFP expression detected here is below the background for this experiment (cf. Fig. 2.6 b and c).

FACS data for five groups of experiments are summarized in a chart (figure 2.7.) The mean expression profile for three experimental parameters is depicted in bars, which differ in color and patterning. The expression profile for transient RMCE among given exchange vectors in the presence of Flp is represented by white bars (group +). The background activity of this experiment without any Flp recombinase is shown as black bars (group -). The product of interaction between heterospecific FRT pairs is described in black/white dashed bars (group x). The reference F_3 - F_{wt} is highlighted in red.

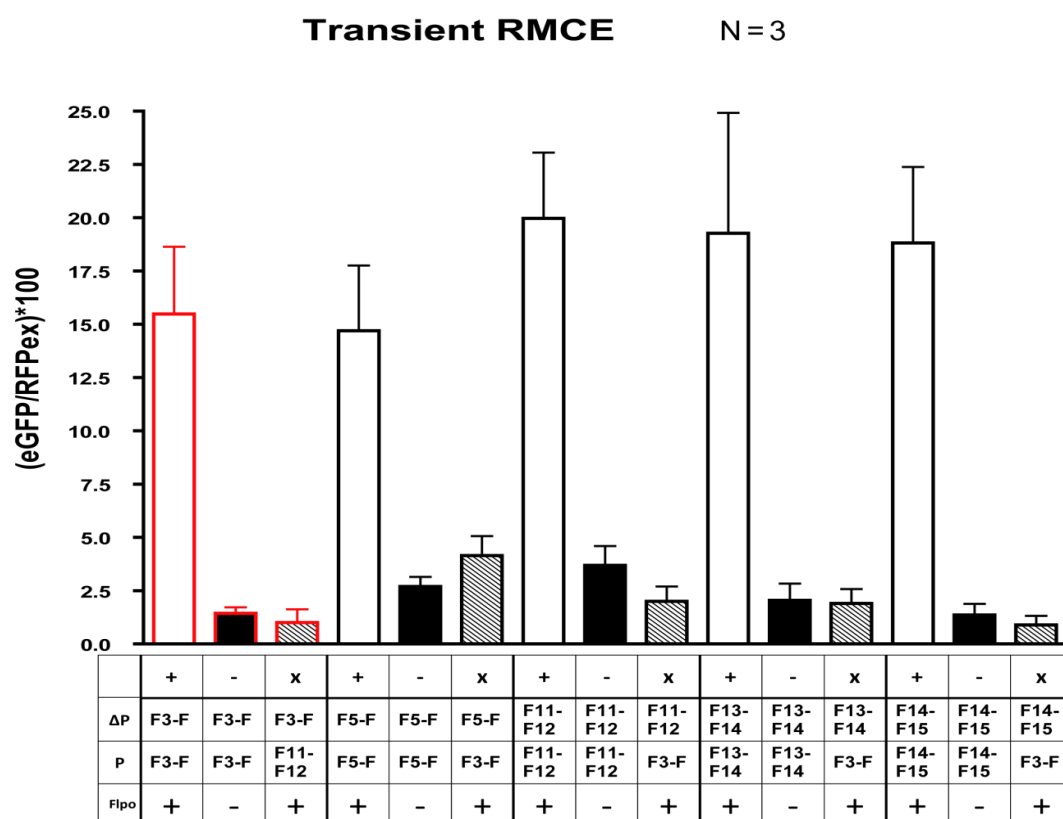


Fig. 2.7 Transient RMCE: evaluation of five FRT pairs by three parameters

In NIH/3T3 cells, transient RMCE activity of cells two days post transfection for all five groups is shown in a summarizing diagram. Three experimental settings were followed:

Transient RMCE of two educts harboring homospecific FRT pairs with application of Flpo (group +).

Transient RMCE of two educts harboring homospecific FRT pairs without application of Flpo (group -).

Transient RMCE of two educts harboring heterospecific FRT pairs with application of Flpo (group x).

Detected eGFP/RFPex coexpression of all groups was normalized to average RFPex expression of group(-) specimen and multiplied by 100. Activity between vectors with homospecific FRT pairs upon application of Flpo-puro is shown as white bars, while activity of identical experiments but without supply of Flpo-puro (background activity) is shown as black bars. In order to detect cross-recombination among vectors with different FRT pairs four combinations F_{11} - F_{12} , F_{13} - F_{14} , F_{14} - F_{15} , and F_5 - F_{wt} of plasmid type ΔP_{gfp} were compared with the conventional version F_3 - F_{wt} of plasmid P_{rfp} . Pair F_3 - F_{wt} of type ΔP_{gfp} was transfected with pair F_{11} - F_{12} of type P_{rfp} . All transfections were conducted with co-delivery of Flpo-puro and are depicted as black/white dashed bars. Graphs are grouped for each evaluated FRT pair, reference F_3 - F_{wt} is highlighted in red bar frames. All samples were analyzed in duplicates. The experiments were repeated twice (N=3). Groups (+) activities are up to 15 - 20 % for all five groups. While reference pairs F_3 - F_{wt} and F_5 - F_{wt} show mild activities around 15 % the remaining groups, show transient RMCE peaking with approximately 20 %. Groups (-) values lie between 1 - 4 %. F_5 - F_{wt} and F_{11} - F_{12} show highest background activity, whereas combination F_{14} - F_{15} and F_3 - F_{wt} show lowest background activity. Cross-recombination activity among both donors, depicted by group (x), is most pronounced in the experiment, in which pair F_3 - F_{wt} is matched with F_5 - F_{wt} with activities of approximately 3.5 %. All other tested pairs, here, show a very low activity for transient RMCE, which is even lower than background activity of the corresponding groups. This suggests no cross-recombination among donors with novel FRT pairs when aligned to a donor with FRT pair F_3 - F_{wt} .

For normalization of the recorded eGFP/RFPex activity transfection efficiency was determined. Therefore, mean RFPex expression of group (-) specimens was determined and used as a transfection control. To normalize determined activities the quotient of (eGFP⁺ cells /RFPex⁺ cells) of all groups (+/-/x) was formed and divided by RFPex⁺ cells of group (-). The product was multiplied by 100. Each sample was analyzed in duplicates and each experiment was repeated twice (N=3).

The average activity of group (+) ranges between 15 and 20 % for all five examined FRT pairs. Reference controls F_3 - F_{wt} and F_5 - F_{wt} show mild activities with approximately 15 % while the other three FRT sets formed of novel mutant FRT sites have a slightly higher activity with approximately 20 % (Fig. 2.7). Remarkably, the experimental variances indicated by error bars are high for all samples belonging to group (+). They are strikingly lower, however, in samples of group (-) or group (x). These given differences of variances might be ascribed to the reversible process of transient RMCE, which occurs at a higher frequency in group (+) and thus might explain fluctuation of the values.

Background activity, determined by group (-), is between 1.4 and 3.7 %. F_5 - F_{wt} and F_{11} - F_{12} show highest, F_3 - F_{wt} and F_{14} - F_{15} lowest activities. Differences among these values cannot be explained.

Group (x), which summarizes cross-recombination potential among pair F_3 - F_{wt} of vector P_{rfp} and F_{11} - F_{12} , F_{13} - F_{14} , F_{14} - F_{15} , and F_5 - F_{wt} of vector ΔP_{gfp} ranges from 0.7 to 4.2 %. FRT pairs F_3 - F_{wt} with partner F_{11} - F_{12} and F_{14} - F_{15} with partner F_3 - F_{wt} show the weakest propensity of cross-recombination, while combination F_5 - F_{wt} with partner F_3 - F_{wt} shows a significant higher activity (4.2 %). This high activity might be due to site specific recombination between homologous variants of wild type FRT sites and between heterospecific FRT site F_3 and F_5 . Recombination among those sites was reported before (Schlake & Bode, 1994) and is likely to occur due to a higher spacer similarity (Tab. 2.2).

All transient RMCE activities of all examined FRT pairs of group (+) were normalized against background activities (group -) and are shown in figure 2.8. The relative fold induction of transient RMCE is seen in white bars and the background, which has been set to 1, is pictured in black bars. The combinations F_5 - F_{wt} and F_{11} - F_{12} show the weakest potential for transient RMCE with a mean 5.4-fold induction.

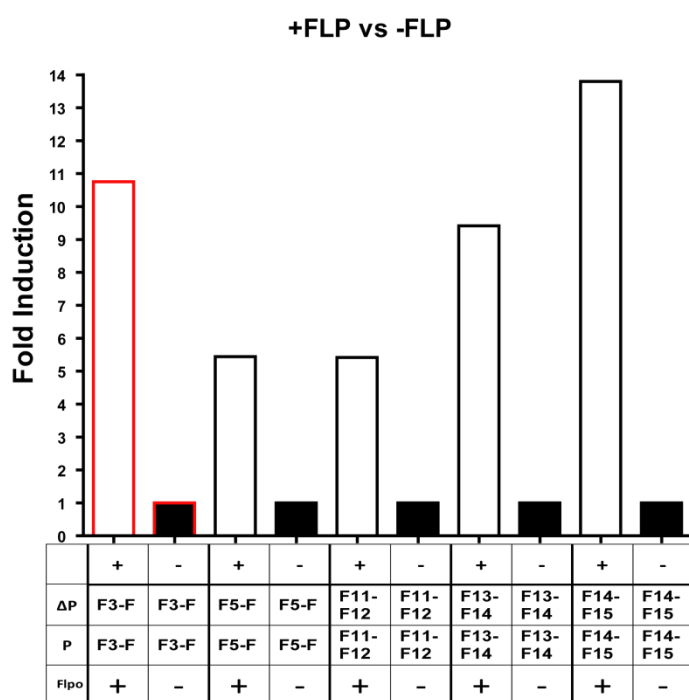


Fig 2.8 Transient RMCE: Comparison +FLP vs -FLP

The relative induction of transient RMCE for five examined FRT pairs is shown. Transient RMCE activity depicted in white bars was normalized versus the measured background activity of the Flp⁻ controls (black bars, value = 1). First row: +/- separate transient RMCE from control experiments. Second row: transfected plasmids of the type ΔP specified by its FRT pair are shown. Third row: transfected plasmids of the type P specified by its FRT pair are shown. Fourth row: cotransfection of Flpo is pictured by + or -. The most active combination is F₁₄-F₁₅ with a 14-fold induction. Red-framed reference F₃-F_{wt} shows a 10.6-fold induction. While F₁₃-F₁₄ shows moderate average values of approximately 9, F₁₁-F₁₂ and F₅-F_{wt} show a weak potential with an induction of 5.4.

Pair F₁₃-F₁₄ shows a moderate potential for transient RMCE with a relative fold induction of ~9, while the reference pair F₃-F_{wt} indicates strong activity with a fold induction of 10.6. The best performance for transient RMCE is clearly seen for FRT set F₁₄-F₁₅ showing a fourteen-fold induction compared to background activity.

2.3 Multiplexing-RMCE

According to the pre-PCR test several combinations of novel FRT mutants showed self-recognition potential and a negligible low propensity to cross-recombination if compared to the conventional heterospecific FRT sites. The functionality of the novel FRTs arranged in exchange cassettes could be clarified in the transient RMCE assays. Next to the reference pair F₃-F_{wt}, the combinations F₁₃-F₁₄ and F₁₄-F₁₅ turned

out to be the best candidates with a high transient RMCE activity, a low background noise and non-cross-reactivity with donor vector F_3 - F_{wt} .

Final aim of this work was to prove the potential of the novel FRT site combinations in a genomic based multiplexing-RMCE assay, in which two genomic anchored cassettes compatible for cassette exchange are subject for RMCE (see overview in Fig. 2.11a). For this approach F_{11} - F_{12} and F_{13} - F_{14} were chosen as potential pairs next to the established one F_3 - F_{wt} . Combination F_{13} - F_{14} showed expression levels above-average in the transient RMCE assay, F_{11} - F_{12} rather weak activities (Fig. 2.8). Thus the latter combination served as a negative control in this setup.

2.3.1 Genomic RMCE with novel FRTs (one target)

One difference between episomal and genomic RMCE might be the restricted accessibility to the integrated parental genomic tag for the donor plasmid in the latter system. This critical parameter could have a major impact in terms of exchange efficacy in a genomic system (Fig. 1.4). Thus another test was necessary to show function and efficiency for RMCE with novel FRT pairs in a genomic background. Two vector systems, on the one hand the parental vector and on the other hand the donor vector with FRT pairs F_{11} - F_{12} or F_{13} - F_{14} flanking exchange cassettes were constructed. These were tested in cells, which had been previously stably tagged with several copies of parental vectors. While transient RMCE data was retrieved by determining gain of fluorescence following a “promoter-outside” strategy (Fig. 2.5) analysis of genomic RMCE events was performed by a more dedicated PCR-based test, which was also applied for multiplexing RMCE studies (ch. 2.3.3).

In order to introduce a primer binding site for PCR analysis of successful targeting, parental constructs were equipped with a 220 bp sequence (depicted as +220 in the constructs' name). 1.0 μ g of *ScaI*-linearized parental vector F_{11} -tkneo- F_{12} +220 or F_{13} -tkneo- F_{14} +220 was electroporated into a wild type clone of NIH/3T3 cells. After electroporation, 2E5 cells were cultured 14 days in G418 selection medium (500 U/mL) on 10 cm plates. Resistant growing cell clones were collected via trypsinization and pooled in order to gain a representative collection of cells with several stably expressing parental vectors in different genomic loci.

To perform RMCE these cells were transfected with 3.0 µg of donor plasmid DNA, for instance F_{11} -RFPex- F_{12} for the target F_{11} -tkneo- F_{12} +220 or F_{13} -RFPex- F_{14} for the target F_{13} -tkneo- F_{14} +220. For each transfection 1.0 µg Flpo-puro was cotransfected, while for negative controls Flpo-expressing vector was replaced by puromycin-expressing construct pBS-pacΔP (ch. 2.2.2). 24 hours after transfection culture medium was replaced with a puromycin containing culture medium (2.5 µg/µl). 48 hours post transfection puromycin resistant colonies were harvested and genomic DNA was prepared. ~300 ng of genomic DNA was subjected to PCR to check for successful gene targeting. In order to demonstrate RMCE one primer binds in the transgenic region of the donor cassette. Another primer anneals in the backbone region of the parental vector. To avoid false positive amplicons the priming regions of the donor must not be on the parental backbone and vice versa. Targeted integration due to RMCE was able to be shown by PCR products of 360 bp size for both evaluated FRT pairs (Fig. 2.9).

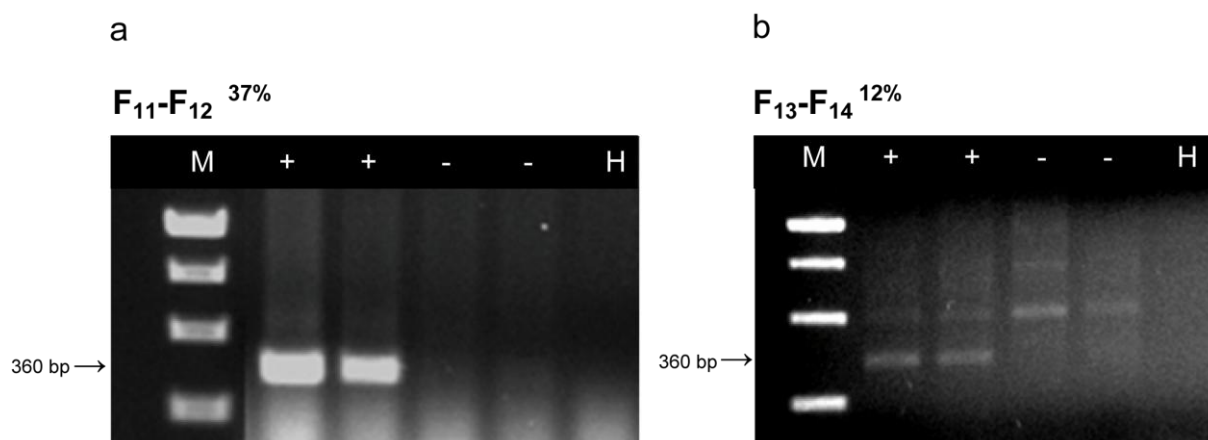


Fig. 2.9 RMCE with novel FRT sites in a genomic background of NIH/3T3 cells

a: PCR analysis after RMCE with FRT-pair F_{11} - F_{12} vectors on a cell pool of a G418 resistant cell line, which was previously electroporated with F_{11} -tkneo- F_{12} +220; PCR band of 360 bp size exemplifies RMCE **b:** PCR analysis after RMCE with FRT-pair F_{13} - F_{14} vectors on a cell pool of a G418 resistant cell line, which was previously electroporated with F_{13} -tkneo- F_{14} +220; PCR band of 360 bp size proves RMCE. +, sample transfected with 3.0 µg of donor plasmids and 1.0 µg of flpo-puro; -, sample transfected with 3.0 µg of donor plasmids and 1.0 µg of neutral vector pBSpacΔP; H, water control. The striking difference regarding band intensities, which does not coincide with transient RMCE data, might be due to different transfection efficiencies, depicted next to the described FRT pair on the top of the figure. Another reason might be the accessibility of the integrated cassettes in the corresponding clones.

Bands depicting occurrence of RMCE could be excised from agarose gels and purified. Obtained DNA was sequenced successfully to clarify cassette exchange (data not shown). Intensities of PCR bands differ clearly with respect to the chosen FRTs. While PCR-amplified band is bright for F_{11} - F_{12} tagged cells it is weak for F_{13} - F_{14} tagged cells in the Flp^+ -samples. The differences in band intensities might be due to different transfection efficiencies for the examined cell lines. While the F_{11} -tkneo- F_{12} +220 pool cell line could be transfected with efficiencies up to 37 % it was only approximately 12% in case of the F_{13} -tkneo- F_{14} +220 pool cell line in a similar experiment. However, for both setups, samples without delivered Flp (Flp^- -samples) show no RMCE specific band.

2.3.2 Construction of multiplexing-RMCE competent cell lines

In order to facilitate construction of multiplexing-RMCE compatible cell lines, a previously used clone was used. This clone, called N1 (Seibler J., Dissertation, TU-Braunschweig, 1999) contains one genomic copy of the parental tag F_3 -hyg tk - F_{wt} . In a former study the hygromycin-thymidine kinase fusion gene (hyg tk) cassette could be exchanged by a donor-delivered β -geo (lacZ-neomycin fusion gene) cassette (Fig. 2.10). Expression level of N1 was shown to be the best of five investigated clones. (Fig. 2.10 adopted from Goetze *et al.*, 2005). N1 was chosen for the construction of multiplexing-RMCE competent cell lines. Cells of N1 were treated with the selective drug hygromycin B (150 U/mL) for maintenance of transgene expression. Clone N1 was provided with additional parental cassettes F_{11} -tkneo- F_{12} +220 or F_{13} -tkneo- F_{14} +220 respectively. These were ScaI -linearized and 1.0 μg of linearized DNA was electroporated to $1\text{E}6$ cells. $1\text{E}5$ cells were seeded on 10 cm plates and 24 hours after electroporation the selective agent G418 (500 U/mL) was added to the culture medium. After 14 days of double selection (hygB/G418) on 10 cm plates resistant colonies were selected.

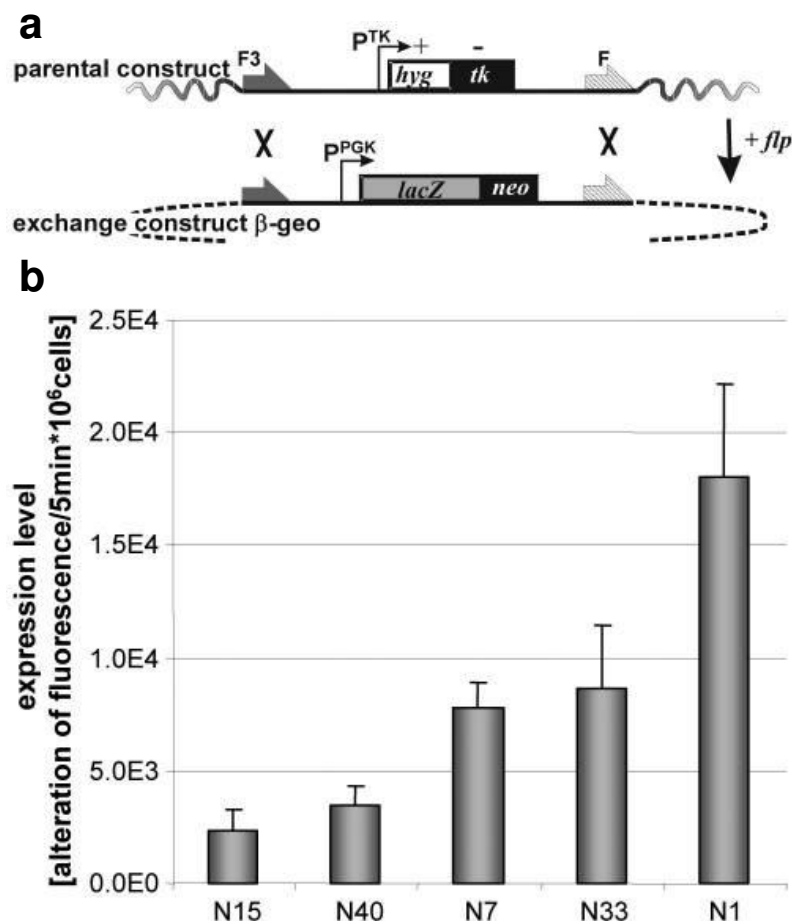


Fig. 2.10 clone N1 shows high β-Galactosidase expression after RMCE

a: experimental setup; the resident single copy cassette harboring a hygromycin-thymidinekinase (hyg^rtk) fusion gene can be replaced by RMCE with a donor bearing a β-geo (lacZ-neo) expression construct. After targeted integration lacZ expression can be determined quantitatively. **b:** the expression of β-Galactosidase for five exchanged clones is depicted. Statistical analysis revealed a significant difference between average expression levels among the loci. While N15 and N40 show expression levels up to 4E3 a five-fold higher expression could be monitored for clone N1, which was chosen for this study. Adapted from Goetze *et al.*, 2005.

Single cells of this pool were seeded into 96 wells by limiting dilution. Growing cell clones were characterized for copy numbers in Southern blot experiments with three different restriction enzymes (Fig. 2.11). Both used parental vectors contain unique restriction sites *ScaI*, *XhoI* and *BamHI*, which were used for Southern Blot bordering-element analysis. Both types of parental vectors were used for electroporation. While Clone N1 electroporated with F₁₁tkneoF₁₂+220 was termed M, N1 electroporated with F₁₃-tkneo-F₁₄+220 was termed O.

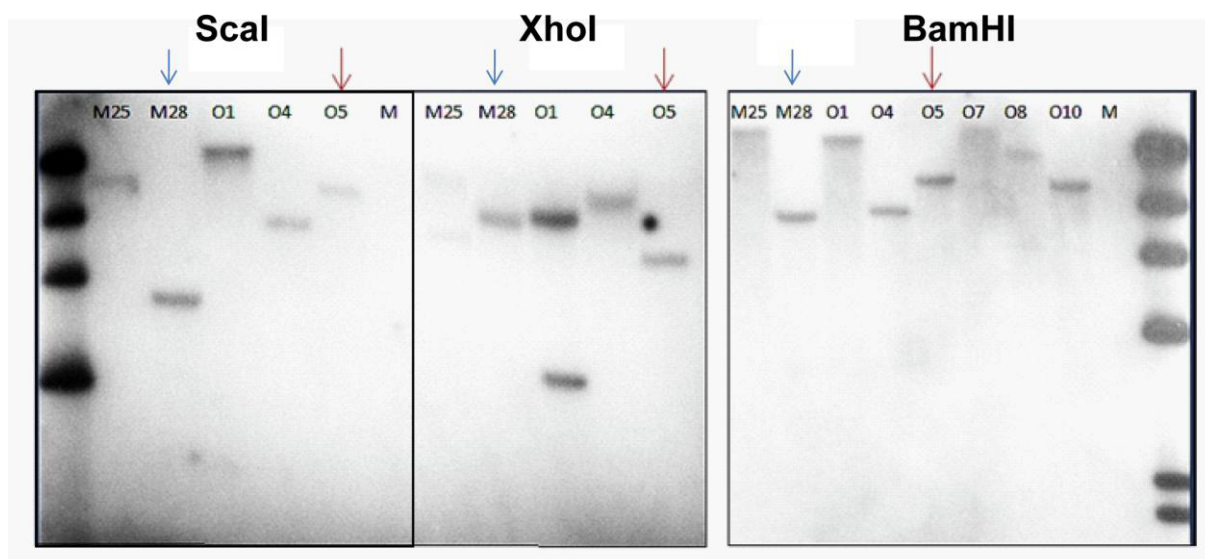


Fig. 2.11 bordering-element analysis for five clones with *Scal* and *XhoI* or eight clones with *BamHI*, respectively.

Clones of the M-series were derived from electroporation of parental vector F_{11} -tkneo- F_{12} +220 into N1 (Fig. 2.10). The O-series was derived from N1 cells, which were electroporated with F_{13} -tkneo- F_{14} +220. Both clone series were treated simultaneously with hygB/G418 selective agents. Resistant single cell clones were isolated by limiting dilution. Southern Blot bordering-element analysis was conducted to show single copy integration of electroporated constructs. cells. A red arrow marks clone O5, which was selected for multiplexing-RMCE studies with the sites F_3 - F_{wt} and F_{13} - F_{14} . O5 shows a single band pattern for all three used restriction enzymes (*Scal*, *XhoI* and *BamHI*) confirming single copy integration of F_{13} -tkneo- F_{14} +220 in N1. Multiplexing-RMCE with the sites F_3 - F_{wt} and F_{11} - F_{12} were conducted with clone M28 (blue arrow), which also shows a single copy integration of F_{11} -tkneo- F_{12} +220 in N1. M: mock wild type NIH/3T3 cell line.

Southern Blot analysis revealed several clones with unique genomic copies. Clone O5 and M28 showed a single band for all three restriction enzymes used and were chosen for final multiplexing-RMCE experiments.

2.3.3 Multiplexing-RMCE with clone O5

There are several options for engineering multiple loci by RMCE. The sequential exchange starts with a targeting of locus 1. After completion locus 2 can be exchanged. After the first RMCE of locus 1 subclones have to be derived from a PCR-positive cell pool in order to find one authentically exchanged cell. The same procedure has to be applied for RMCE at locus 2. An alternative approach is the simultaneous targeting of both loci in a single transfection. In order to meet this aim, re-

combinases-mediated cross-interaction with donor vectors should be excluded. Transient RMCE data showed no indication of significant cross-recombination events occurring in case two episomal donor vectors were present in parallel (Fig. 2.7).

While a sequential exchange seems to be beneficial in terms of efficiency of each step, negative selection can only be applied for enrichment of exchanged clones in a parallel exchange setup.

The simultaneous exchange of both loci was addressed for clone O5 (Fig. 2.12). 24 hours before transfection 2E5 cells were seeded on five 6 well plates for each experiment. 4 hours before transfection culture medium was changed for a medium containing no selection drugs. 1.5 μ g of both donors F₃-cmv-d2eGFP-F_{wt} and F₁₃-RFPex-F₁₄ were transfected together with 1.0 μ g of Flpo-puro according to the Metafectene® transfection protocol. As a negative control Flpo-puro was substituted by empty DNA pBS-pac Δ P. 24 hours after transfection medium was replaced by puromycin (2.5 μ g/mL) containing DMEM to start selection of transfected cells. 48 hours post transfection cells were detached, pooled by trypsinization and sorted twice for both fluorescent markers on a XDP FACS machine within seven days. Post second sorting cells were cultured on 6 wells in DMEM medium. Upon reaching the level of confluency a part of the cells was used for initial PCR analysis, the other fraction was subjected for negative selection steps.

Semiquantitative PCR was conducted to gain evidence for both cassette exchange reactions in a given cell pool. In figure 2.12 b and c. PCR-amplified products are depicted in an electrophoretic 1 % agarose gel analysis. A broad distinct band of 626 bp size shows targeted integration of F₃-cmv-d2egfp-F_{wt} donor in locus F₃-hyg^{tk}-F_{wt}. The amplification of a weak band of 360 bp size proofs targeted integration of donor F₁₃-rfpex-F₁₄ into locus F₁₃-tkneo-F₁₄+220 (Fig. 2.12 b). The difference in the band intensities might be due to accessory limitations of the donors to their corresponding parental cassettes. The weak band of the F₁₃-F₁₄ locus might be also ascribed from a weaker potential for RMCE compared to the F₃-F_{wt} pair (Fig. 2.8).

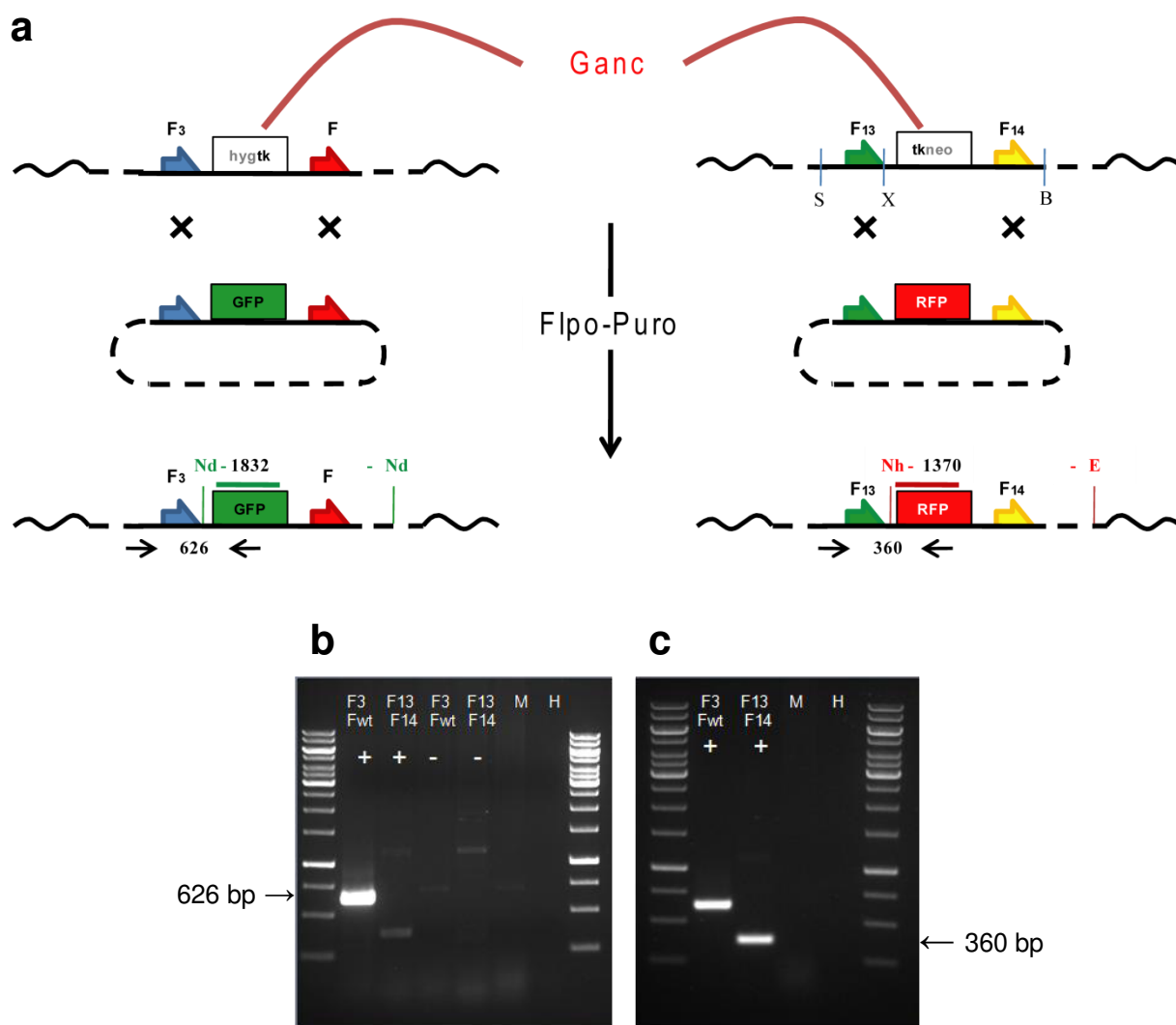


Fig. 2.12 Schematical sketch of multiplexing RMCE at two distinct loci

a: Clone N1, which harbors one copy of F_3 hygtkF_{wt} was used as a template cell line for introducing a second cassette F_{13} tkneoF₁₄+220. Single copy status of the construct had been verified from bordering fragments yielded by Southern blotting with three different enzymes (S ; ScaI, X; XhoI and B; BamHI)(Fig. 2.11). PCR and Southern blotting was used to verify RMCE. Successful targeting could be shown with PCR by amplification of a 626 bp fragment for the locus F_3 -F_{wt} and 360 bp for the locus F_{13} -F₁₄. Primers are indicated by horizontal arrows. Verification of RMCE by Southern Blot was feasible by obtaining fragments of specific size (1832 bp/ F_3 -F_{wt}; 1370 bp/ F_{13} -F₁₄) after enzymatic digestion of the genomic DNA (NdeI (Nd) / F_3 -F_{wt}; EcoRI (E),NheI (Nh) / F_{13} -F₁₄). Fragments were visualized with a radioactively labeled *gfp* or *rfpex* probe. **b:** Both cassettes could be subjected to RMCE by cotransfection of 1.5 μ g donor vectors and 1.0 μ g Flpo-puro. Targeted integration by Flp-RMCE of both plasmids was determined by PCR-derived bands of 626 bp for the F_3 -F_{wt} locus and 360 bp for F_{13} -F₁₄ locus. No amplification of specific bands was obtained if Flpo-puro was replaced by control DNA, M: untreated clone O5, H: water control. **c:** Intense signals indicating RMCE for both loci. Cells could be enriched after 14 days of negative selection of doubly sorted cells with Ganciclovir. M: untreated clone O5, H: water control.

This experiment was performed in the same way with cell line M28, harboring one copy of the F_3 - F_{wt} and the F_{11} - F_{12} parental locus (Fig.2.11). After transfection and two sorting rounds no PCR band corresponding for targeted integration of donor F_{11} -rfpex- F_{12} could be revealed (data not shown).

Both parental vectors bear a positive/negative selection marker fusion gene, which is hygromycin-herpes simplex virus thymidine kinase (*hygthk*) for the locus F_3 - F_{wt} and herpes simplex virus thymidine kinase-neomycin (*tkneo*) fusion gene for the locus

Tab. 2.3 Negative selection schedule of clone O5

2E4 cells (1 x and 2 x sorted) with and without codelivery of Flp were seeded on 10 cm plates. Cells were treated with three (100 nM, 1000 nM, 3000 nM) different concentrations of Ganc. A significant number of 50 clones were detected on doubly sorted Flp⁺-samples treated with 100 nM Ganc. No clonal growth was detected in the according Flp⁻-controls. Flp⁺-samples treated with a 10-fold or 30-fold higher concentration of Ganc did not show any clonal growth either. Negative selection of untreated O5 control gave rise to three colonies, which might be due to spontaneous resistance.

Forming clones after transfection, sorting and negative selection of clone O5			
concentration Ganc in nM	100	1000	3000
Flp ⁺ (1 x sorted)	3	0	0
Flp ⁻ (1 x sorted)	0	0	0
Flp ⁺ (2 x sorted)	~50	0	2
Flp ⁻ (2 x sorted)	0	0	0
Untreated clone O5 (control)	3	0	0

F_{13} - F_{14} (Fig. 2.12 a). Thymidine kinase (tk) converts the prodrug Ganciclovir (Ganc), a non-toxic nucleoside analogue of 2'-deoxy-guanosine, into its triphosphate analogue form deoxyguanosine triphosphate (dGTP). By incorporation of this analogue into elongating virus DNA, elongation is terminated and cell death will be triggered (Rubsam *et al.*, 1999).

Negative selection is feasible for the enrichment of doubly exchanged cells. Since replacement of both target cassettes by corresponding transgene sequences from

donors, no tk unit remains anchored genomically. Thus only cell populations, for which both loci were targeted, survive the treatment of Ganciclovir.

2E4 cells of a fraction of O5 doubly sorted eGFP/RFPex positives was seeded on 10 cm plates and treated with Ganciclovir (100 nM) for 14 days. In the Flp⁺-derived population Ganc-resistant colonies appeared after several days of selection, whereas no clones appeared in the Flp⁻-control (Tab. 2.3). Flp⁺-populations, which were sorted once showed no clone growth at all. This suggests a necessity for two sorting steps in a multiplex targeting assay. Future solutions will imply the promoter-outside strategy as discussed under chapter 3.6.2. The survival of three colonies of untransfected clone O5 has likely been the result of spontaneous resistance (personal communication with Qiao, J. and Oumard, A.).

Selection was finished by trypsinization and pooling of the grown clones. Genomic DNA was prepared and PCR was performed to indicate exchange for both loci (Fig. 2.12 c). Both bands, 626 bp for the locus F₃-F_{wt} and 360 bp for the locus F₁₃-F₁₄ show gene targeting. The strength of the band derived from the F₁₃-F₁₄ locus is comparable to its F₃-F_{wt} counterpart and superior to the PCR amplicon of the non-Ganc-treated samples (cf Fig. 2.12 b with c). Thus, Ganc-mediated negative selection benefitted and significantly increased the efficiency of targeting, especially at locus F₁₃-F₁₄.

In order to show the double exchange at both loci on a single cell level 29 clones were recovered from the Ganciclovir resistant cell population (Flp⁺ 2x sorted) of clone O5 (Tab. 2.3) by limiting dilution. Genomic DNA of all 29 subclones was prepared and subjected to PCR for confirmation of RMCE at both loci.

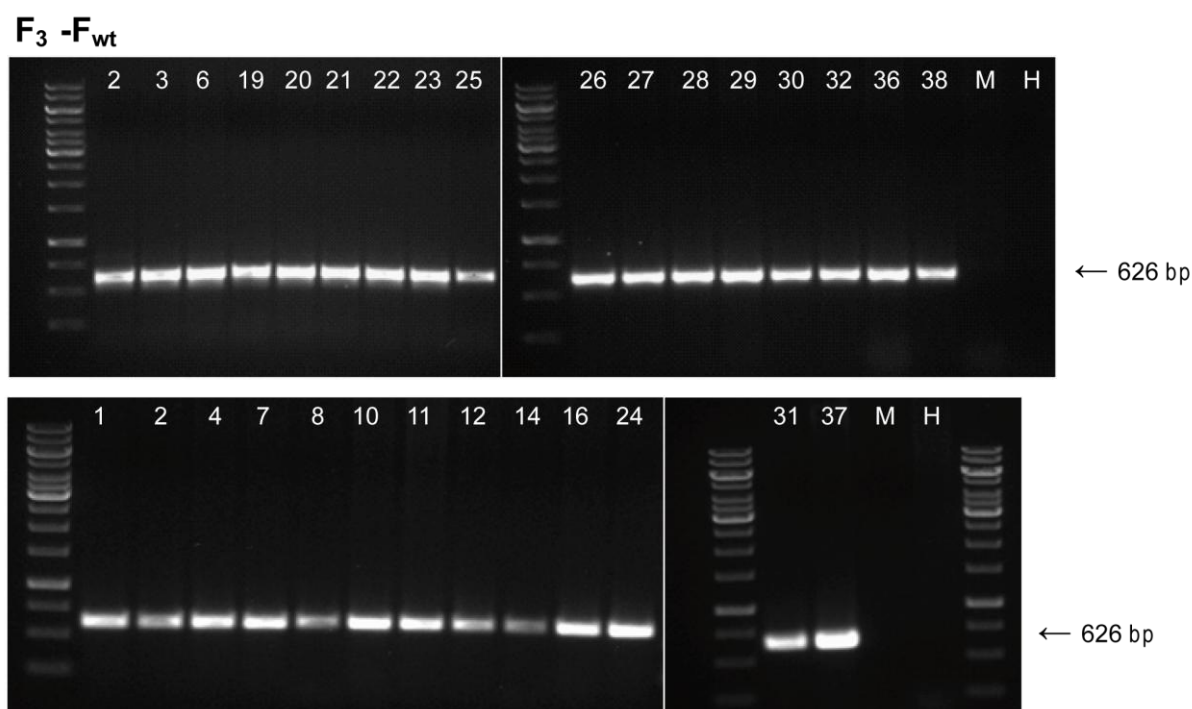


Fig. 2.13 Confirmation of targeted integration of donor plasmid into locus F_3 - F_{wt} by PCR in NIH/3T3 cells

29 single cell clones of the Ganc-resistant pool population (Fig 2.12 c, Tab. 2.3) were isolated by limiting dilution and analyzed for gene targeting. PCR-amplified 626 bp fragment in all 29 clones shows targeted integration of the FRT flanked donor sequence into the addressed locus F_3 - F_{wt} . All bands have a comparable density. Untreated clone O5 (M) and H_2O (H) are blank. The targeting efficiency is 100 % for this locus of clone O5.

Electrophoretic gel analysis shows RMCE for both loci in all evaluated clones (Fig. 2.13, 2.14). This data underlines the feasibility of multiplexing-RMCE in NIH/3T3 cells in a single transfection step with targeting efficiency up to 100 % for both loci of clone O5. While PCR is a helpful tool to show RMCE, random integration of donor plasmids cannot be uncovered with that technique. In order to both confirm RMCE and to show random integration events, Southern Blot analysis of 19 clones for RMCE at locus F_{13} - F_{14} and 10 clones for RMCE at locus F_3 - F_{wt} of O5 were carried out (see overview: Fig. 2.12 a). Targeted integration of F_3 -cmv-d2egfp- F_{wt} donor into the given locus of clone O5 could be shown after digesting genomic DNA with NdeI. This restriction enzyme cuts once in the original backbone and once in the integrated donor plasmid sequence, giving rise to a 1832 bp band. The fragment contains an *egfp* (*d2egfp*) ORF, which could be detected with a radioactive labeled *egfp* probe. 8 of 9 analyzed clones show a characteristic band for RMCE at the F_3 - F_{wt} locus (Fig. 2.15 a). In all of

these clones no additional band of 3130 bp, which indicates random integration of the F_3 -cmv-d2egfp- F_{wt} donor plasmid, was noticed.

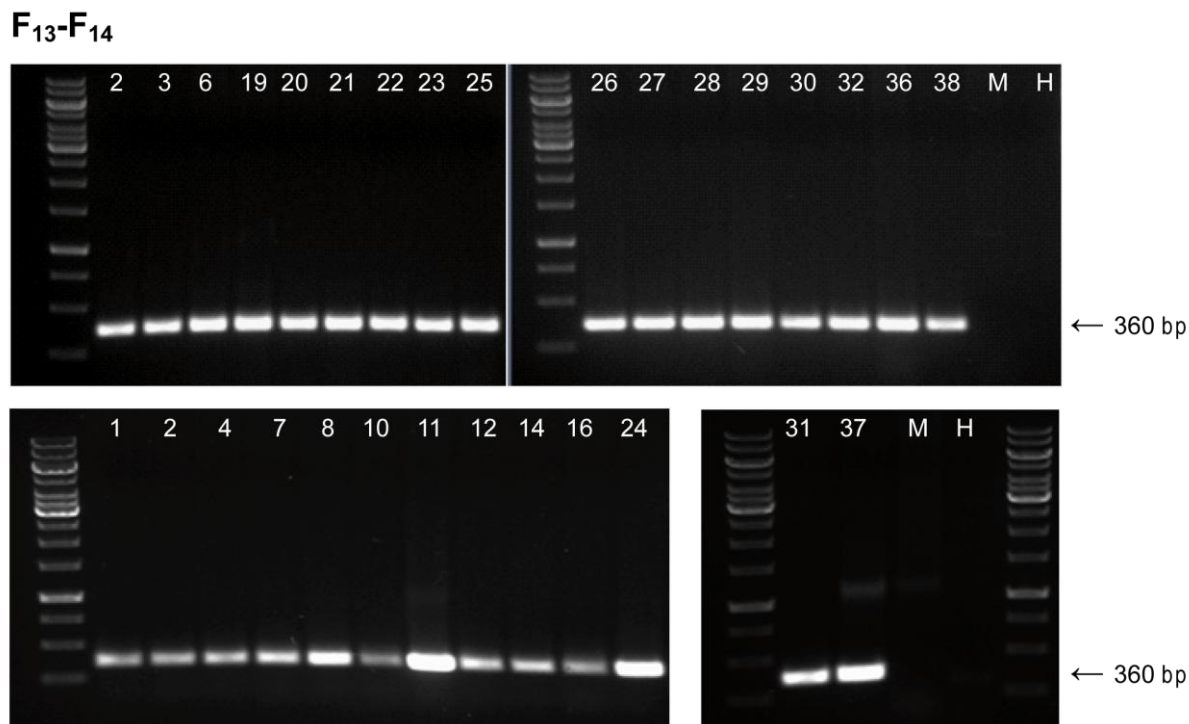


Fig. 2.14 Confirmation of targeted integration of donor plasmid into locus F_{13} - F_{14} by PCR in NIH/3T3 cells

29 single cell clones of the Ganc-resistant pool population (Fig 2.12 c, Tab.2.3) were isolated by limiting dilution and analyzed for gene targeting. PCR-amplified 360 bp fragment is detected in all 29 clones and indicates targeted integration of the F_{13} - F_{14} flanked donor sequence at the locus F_{13} - F_{14} . All bands have a comparable density. Untreated clone O5 (M) and H_2O (H) are blank.

RMCE with pair of sites F_{13} - F_{14} could be shown with EcoRI/NheI double digestion of the genomic DNA. EcoRI cuts once in the backbone of the parental plasmid and NheI once in the integrated donor sequence. A band of 1370 bp is specific for F_{13} - F_{14} RMCE and could be visualized with a radioactively labeled *rfpex* probe (Fig. 2.15 b and b2). 18 out of 19 clones show a band of 1370 bp. There is no indication of randomly integrated donor plasmids F_{13} -*rfpex*- F_{14} , which would be revealed by a 2048 bp long band. Clone 19, however, showed a larger band but none matching for RMCE or random integration at both loci

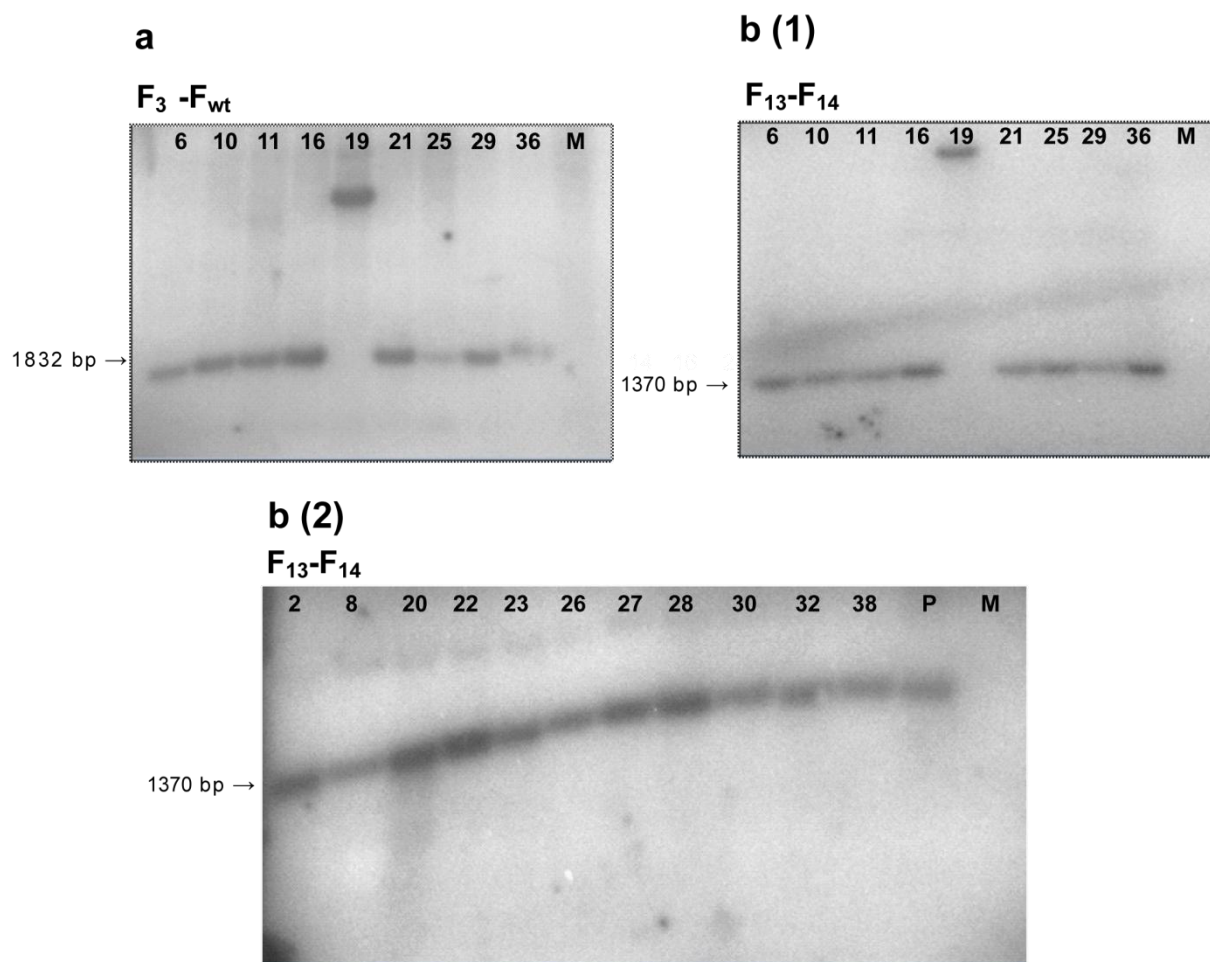


Fig. 2.15 Confirmation of targeted integration of donor plasmid into loci F₃-F and F₁₃-F₁₄ by Southern Blot

a: 8 out of 9 clones show a band at a size of 1832 bp, which indicates RMCE at the parental F₃-F locus. Genomic DNA was digested with NdeI, cutting in the backbone of the parental plasmid and in the integrated donor sequence. Randomly integrated donor plasmids could be ruled out, because no band appeared at 3130 bp. Membrane was hybridized with an *egfp* probe. Clone 19 showed no band indicating RMCE or random integration but a band at a larger size. **b(1)** and **b(2):** 18 of 19 analyzed single cell clones and the cell pool (P) show evidence for RMCE at the genomic locus F₁₃-F₁₄. The 1370 bp fragment, yielded by cutting with EcoRI in the backbone of the parental plasmid and NheI in the FRT flanked donor sequence, could be visualized by a radioactive probe of the *rfpex* ORF fragment. No patterning of random integration, which would result in a 2048 bp band was detected in all clones. M, Mock control= untreated clone O5

Both bands seemingly have the same size suggesting illegitimate recombination among both donor vectors F₃-d2egfp-F_{wt} and F₁₃-rfpex-F₁₄. Due to some larger regions of sequence homology between both vectors this scenario is comprehensible.

This explanation seems to contradict the previously shown PCR data, which confirms RMCE for both given loci (Fig. 2.13; Fig. 2.14). This phenomenon could be explained

by raise of intermediate products, which might have occurred by initial recombination of $F_{13} \times F_{13}$ or $F_3 \times F_3$ respectively. Intermediates during RMCE were proposed by Schlake & Bode, (1994), and might give rise to a different restriction pattern, which complies to Southern Blot data (Fig. 2.15). Yet, likelihood of such events is very low and it is unlikely that this incomplete RMCE happened to both loci simultaneously.

2.3.4 Targeted clones show double expression of fluorescent markers

All 29 single cell clones obtained from negatively selected Flp^+ 2x sorted samples (Tab. 2.3) showed expression of both fluorescent markers in FACS analyses. A selection of five clones is pictured for RFPex and d2eGFP expression (Fig. 2.16).

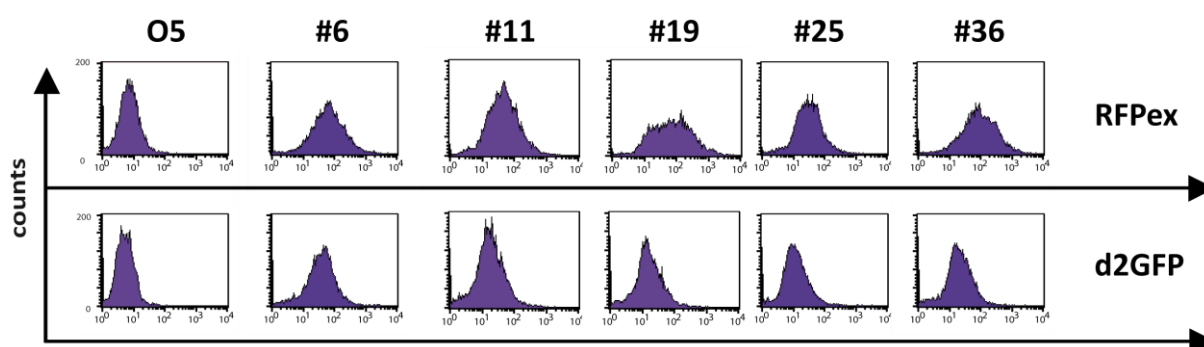


Fig. 2.16 FACS expression histogram of 5 doubly exchanged clones and untreated clone O5

Vertical axis shows cell count (0 - 200), horizontal axis shows d2eGFP and RFPex expression on a logarithmic scale. All doubly targeted sub clones are derived from a Ganc-resistant pool population (Tab. 2.3). All clones show transgene expression above background level (control: clone O5). However, the heterogenous expression pattern of the subclones does not fully reflect clonal nature for most clones.

Expression level of all clones exceeds background expression of control (clone O5). Expression of marker RFPex seems to have a higher mean expression than d2eGFP, even though with a higher variance. One reason for the low expression level of d2eGFP might be the determined half life of 2 hours (Li *et al.*, 1998), whereas RFPex half life was reported to be at least 4.6 days (Mirabella *et al.*, 2004). The differences in stability of the expressed proteins might reflect expression characteristics here.

Expression of derived clones appears to be heterogeneous and shows no characteristic peak-like pattern, suggesting clonality (cf. Fig 2.17).

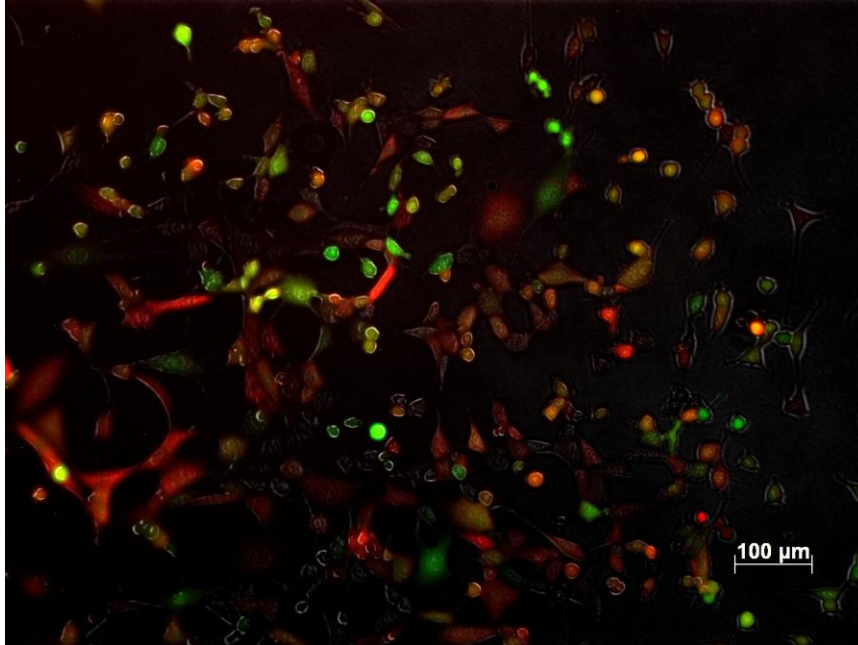


Fig. 2.17 Fluorescence microscopy of Flp⁺ 2x sorted cells of a Ganc-resistant pool population from clone O5

d2eGFP fluorescent (green) cells indicate targeting of the locus F_3 - F_{wt} , while RFPex fluorescent (red) cells imply targeting of the locus F_{13} - F_{14} . d2eGFP and RFPex fluorescent cells (orange and yellow) cells, however, suggest simultaneous targeting of both loci. Expression of both fluorescent markers appears heterogeneous in cells. While this appears to be likely for a pool population similar expression pattern were seen in pool-derived sub clones (Fig. 2.16).

This is unexpected, since clones with the same genomic integration pattern should be isogenic. Nevertheless, similar studies, in which isogenic subclones were derived of targeted clones, also confirmed a heterogeneous transgene expression pattern (Nehlsen, K., unpublished data). Differences in expression patterns of isogenic cells might be for instance due to epigenetic changes in the derived sub clones.

3 Discussion

Site specific recombination (SSR) systems as the FLP/FRT system are versatile tools for genomic engineering tasks. FLP-SSR can be applied for modification of specific genomic regions by excision, inversion or the introduction of DNA cassettes (Bode *et al.*, 2000b). A more advanced technique is the FLP-recombinase-mediated cassette exchange (FLP-RMCE) first shown by Schlake & Bode in 1994. RMCE permits the repeatable modification of a previously established genomic locus by the precise exchange of a DNA fragment, which is flanked by two heterospecific FLP recognition target sites (FRT), (review: Oumard *et al.*, 2006).

This work exemplifies the effective expansion of the RMCE technology by the introduction of several novel sets of FRT sites (initial work: Turan, S., diploma thesis, TU-Braunschweig, 2007). These novel sites were designed according to several rules. After construction, two tests of different complexity critically evaluated the potential of these FRTs in terms of recognition potential with identical partner sites (“self-recognition”) and inhibition of recombination among heterospecific FRTs (“discrimination”). Together with the prominent pair F_3 - F_{wt} , the combination of sites F_{13} - F_{14} but not F_{11} - F_{12} was found to be successfully applicable for (multiplexing)-RMCE for simultaneous targeting of two loci in NIH/3T3 cells.

3.1 Construction of valid novel FRTs has to meet three criteria

3.1.1 48 bp FRTs rather than 34 bp minimal sites are required for RMCE

Umlauf & Cox, (1988) used 34 bp minimal FRT sites consisting of two inverted repeats, flanking an 8 bp spacer, for their studies. These sites show a high potential for intramolecular recombination (excision) but a very low one for intermolecular recombination (integration) events in prokaryotes and eukaryotes (Lyznik *et.al.*, 1993). For RMCE, recombination in *trans* is an essential step, which can be solely guaranteed by 48 bp FRTs, composed of three repeats (Fig. 2.1). These were shown to serve as a substrate for integration reactions, whereas minimal 34 bp FRT sites

were no substrate (Jayaram M., 1985). One model envisages the third repeat b to enhance the chance for forming a FLP-complex. O'Gorman *et al.*, (1991) used genomically anchored 48 bp FRTs to introduce plasmidial DNA into these loci, which is referred to as basic "Flp-in" principle and is commercially available (Liu *et al.*, 2006). These observations reinforced the concept that three-repeat FRTs have to be applied for the construction of novel FRT mutants.

3.1.2 A functional mutant FRT is defined by three structural features as proposed by Umlauf & Cox, (1988)

FRTs referred to as heterospecific FRTs differ in their 8 bp spacer sequence compared to the wild type FRT. The spacer is defined as a region of recombination involving DNA-DNA pairing, cut and rejoining, which results in a mutual exchange of DNA strands (Andrews *et al.*, 1985; McLeod *et al.*, 1986; Senecoff & Cox, 1986). The spacers' asymmetrical structure defines the orientation of the FRT site and determines the mode of recombination. Two FRT sites with discriminative spacers regarding length and (more important) sequence have a reduced ability to recognize each other and do have an impaired recombination potential (Senecoff & Cox, 1986). In 1988 Umlauf & Cox conducted a study to unravel the structural features of a spacer sequence *in vitro*, which has to be given for a functional minimal 34 bp FRT site (Jayaram, M., 1985). These features (rules) are summarized in Tab.2.1 and were used as a guideline to construct the novel mutant FRTs presented in this work.

3.1.3 The "4 bp rule" revealed by Schlake & Bode, (1994) is necessary for obligate heterospecificity between FRTs

The ability of a FRT to recognize a partner site depends on the homology of the spacer sequences for both sites: the higher the homology, the bigger are the chances for recombination between the FRTs (Senecoff & Cox, 1986; Schlake & Bode, 1994). Vice versa, the higher the discrimination between both spacers, the weaker is the recombination potential. Nevertheless, promiscuous recombination among mutant FRTs was still detected, even if the spacers differed in 1 - 3 bp. Therefore, a

difference of 4 bp between the spacer sequences is needed to reduce cross-recombination to a negligible low level. Mutant FRTs F_3 and F_5 differ in 4 positions with regard to the F_{wt} spacer. They proved to show negligible low cross-recombination with F_{wt} and a sufficient self-recognition potential (Schlake & Bode, 1994). All new mutant FRTs (F_{11} , F_{12} , F_{13} , F_{14} and F_{15}) were constructed obeying most rules concerning function and safety (Tab.2.1, Tab 2.2).

3.2 Characterization of novel FRTs

3.2.1 The PCR test permits detection of recombinational activity among homologous and heterospecific FRTs

Besides the three established FRTs F_{wt} , F_3 and F_5 five novel FRTs were analyzed in two critical tests in order to evaluate their ability regarding self-recognition and cross-recombination. The PCR test made use of recombinational activity among both FRT-tagged vectors and could be conveniently evaluated by PCR. Due to the fact that each vector A or B harbors one specific (mutant) FRT, comparison of all FRTs regarding self-recognition and cross-recombination was straightforward (Fig. 2.2). Related tests were described by Umlauf & Cox, (1988), in which two supercoiled plasmids, each with one FRT, were subjected to addition reactions *in vitro*. After recombination, products were analyzed by restriction patterning.

Other assays made use of altered gene expression after recombination. Here, heterospecific lox variants of the Cre-SSR system flanked an *egfp* reporter to determine the cross-recombination potential. Expression of the reporter was driven by a promoter adjacent to the 5' lox site. A *lacZ* ORF was placed in 3' position to this construct. Upon Cre-mediated excision, constitutive eGFP expression was abolished while β -Galactosidase expression was triggered, due to the spatial rearrangement of the promoter (Siegel *et al.*, 2001). This method reflects the true genomic potential of several mutant sites. Since for each comparison new cell lines have to be established in a laborious way, this concept was disregarded for the present work.

As a straightforward solution, the PCR test established by Schlake & Bode, (1994) was applied. The authors created five FRT variants, called F₁-F₅ by mutagenizing the FRT wild type spacer (Tab. 3.1).

Tab. 3.1 Comparison of the spacer sequence of five FRT mutants (Schlake & Bode, 1994) with F_{wt}. Spacer mutations are marked red for each mutant FRT and number of mutations is listed on the right column. F₃ and F₅ (bold) are mutated in 4 positions and could successfully be tested in RMCE studies (Qiao *et al.*, 2009; Sandhu *et al.*, 2010).

Mutant abbreviation	Sequence	Number of bp mutations compared to F _{wt}
F _{wt}	5' TCTAGAAA 3' 3' AGATCTTT 5'	0
F ₁	5' TCTAGATA 3' 3' AGATCTAT 5'	1
F ₂	5' TCTACTTA 3' 3' AGATGAAT 5'	3
F₃	5' T TC AAATA 3' 3' A AGT TTAT 5'	4
F ₄	5' TCTAGAA G 3' 3' AGATCTT C 5'	1
F₅	5' T TC AA AA G 3' 3' A AGT TT TC 5'	4

Some mutants were reported in former studies. Mutant F₁ equaled pJFS40 (Umlauf & Cox, 1988a) and the F₄ construct corresponded to Mu5T2 (McLeod *et al.*, 1986). Comparison of these sites, which differed in one or two positions relative to the F_{wt}, showed traceable cross-recombination. The generation of new mutants (F₂, 3 bp spacer difference versus wild type; F₃ and F₅: both 4 bp spacer difference versus wild type) with more discriminative spacers compared to F_{wt} site was obligatory to eliminate promiscuous recombination events among compared sites. As expected, FRTs F₃ and F₅ both showed good performance for self-recognition and sufficient inhibition of cross-recombination with wild type FRTs (Schlake & Bode, 1994). These

sites could be successfully used in combination with F_{wt} (F_3 - F_{wt} and F_5 - F_{wt}) for RMCE in several studies (Schlake & Bode, 1994; Qiao *et al.*, 2009; Loew *et al.*, 2009; Sandhu *et al.*, 2010) underlining the importance of the PCR test, in order to find valid FRT combinations for RMCE.

3.2.2 Mutants F_{13} , F_{14} and F_{15} are functional and safe

F_3 interrupts the 5'-polypyrimidine tract once (violation of rule 1, Tab. 2.1), whereas F_5 has a mutated margin base pair (violation of rule 2). In genomic RMCE projects, both pairs were used, in which emphasis clearly shifted in favor of F_3 - F . Even though both combinations are principally applicable for RMCE in a mammalian environment, transient studies revealed that F_3 - F_{wt} works better than F_5 - F_{wt} (Fig. 2.8). Due to this, F_3 - F_{wt} was chosen to be the first pair of sites for implementing a multiplexing-RMCE scenario. This was critically respected for design and construction of novel FRTs.

F_3 served as a model and most of the novel mutant FRTs were constructed critically following rule 2 and 3 (Tab. 2.1). Rule 1 was violated in all new mutants except F_{12} . PCR test analyses revealed good self-recognition of mutants F_{13} , F_{14} , F_{15} , which was comparable to the capacity of F_{wt} and F_3 . However, mutants F_{11} , F_{12} and F_{16} showed an impaired potential for self-recognition, seen in densitometrical analysis of these sites (Fig. 2.3). Impaired recognition by F_{11} might be explained by the three-fold violation of rule 1, whereas the reduced efficacy of F_{12} cannot be explained, as it appears to comply with the standard rules. A second experiment revealed that all mutants did not show any recognition by F_{wt} . This observation confirms importance of the “4 bp rule” (ch. 3.1.3).

3.2.3 Transient RMCE: A practical tool for development and elucidation of novel RMCE vectors

Subsequent to the results of the PCR test (ch. 2.1), questions concerning performance of these sites in a RMCE-like setting arose. A disadvantage of the PCR test is that it presents a semi quantitative method for detecting recombination events. It

might thus not reflect the exact level of Flp-mediated SSR, also due to the fact that there is a length bias between the PCR products (3000 bp vs. 250 bp). In addition to the mentioned points, a critical difference of both tests is that the PCR approach compares single FRTs in terms of self-recognition and cross-recombination potential. Transient RMCE, however, evaluates performance of FRT pairs in a RMCE-like setting (presence on two plasmids). Besides, the PCR test merely checks recognition or non-recognition among FRTs which can lead to integration (fusion) of participating plasmids (Fig. 2.2). It does not cover the intramolecular excision step, which is the second step completing RMCE. In contrast, transient RMCE investigates if a pair of FRTs is capable for cassette exchange, which involves the subsequent steps of integration and excision, according to a model, in which intermediates are formed during the process of RMCE (Schlake & Bode, 1994). So far it remains to be determined if the excision or integration is favored by mutant FRTs relative to the F_{wt} site.

Transient RMCE was applied to uncover the ultimate FRT combinations for the development of novel RMCE cassettes. It was first introduced by Seibler & Bode, (1997). They compared the potential of transient RMCE in different cell lines in a quantitative way. Analogous to the transient approach described in this work they used two vector classes P_{SEAP} and ΔP_{fluc} (Fig. 2.2) P_{SEAP} comprised the reporter secreted alkaline phosphatase (SEAP), and a *hyg^r* fusion gene replacing the *rfpex* gene for the model P_{rfp} (ch. 2.2.1) used in this work. Instead of harboring the *egfp* gene, ΔP contained a promoterless firefly luciferase-IRES-puromycin complex, whose expression is activated upon transient RMCE. Here, fluorescent reporters were used for simplifying read out of data.

Goetze *et al.*, (2005) showed that the expression potential of genomic loci being flanked by insulator elements is uncoupled from effects of the chromosomal surrounding. It was concluded that shielding effects from these elements neutralize positive or negative influences on expression arising from the genomic vicinity. In contrast to genomic RMCE, transient RMCE seems to be uncoupled from chromosomal effects that can affect the expression potential of the gene of interest and also change the recombination efficiency between two recombinase recognition target sites (Vooijs *et al.*, 2001). It also circumvents effects, which limit the accessibility of a

genomic locus being, for instance, heterochromatization or other local or nucleosomal variations.

A handicap that emerged for transient RMCE is the reversibility of the reaction (Fig. 2.5). (Educts: SV40-F'-*rfpex*-F $\times$ F'-*egfp*-F $\rightleftharpoons$ Products: SV40-F'-*egfp*-F $\times$ F'-*rfpex*-F). Under presence of Flp products are able to mediate cassette exchange, which gives rise to educt vectors (indicated by both reaction arrows in the formula). Seibler & Bode (1997), however, described for their transient RMCE experiments transfection of equimolar amounts (2 μ g) of educts P_{SEAP} and Δ P_{fluc} each and 1 μ g of Flp-expressing vector pOG44 for triggering transient RMCE. This equimolar ratio for the Δ P_{gfp}/P_{rfp} system did not work in the present setting. An educt ratio of 1/1 of both vectors did not yield a significant difference among Flp⁺- and Flp⁻-samples (data not shown). Shifting the educt ratio P_{rfp}/ Δ P_{gfp} to 3/1, however, gave rise to data exemplified in Fig. 2.6. Seemingly, the forming equilibrium between both vector-classes during transient RMCE is dependent on the ratio of used educt vectors.

3.2.4 Transient RMCE results are in favor of combinations F₁₃-F₁₄ and F₁₄-F₁₅

For multiplexing-RMCE with sets F₃-F_{wt} // F_x-F_y the “4 bp rule” had to be expanded to all FRTs involved in a given system (F₃, F_{wt}, F_x and F_y). Pairs F₁₁-F₁₂, F₁₃-F₁₄ and F₁₄-F₁₅ that obeyed these prerequisites were constructed and tested for transient RMCE. Results from the PCR test indicated FRT sites F₁₃, F₁₄ and F₁₅ to be most appropriate for further RMCE studies (ch. 3.2.2). Here, developed exchange cassettes with pairs F₁₃-F₁₄, F₁₄-F₁₅, and conventional F₃-F_{wt} all showed similar high induction rates of transient RMCE and had a very low background activity suggesting novel pairs F₁₄-F₁₅ and F₁₃-F₁₄ to be optimal for further genomic RMCE studies concerning activity (Fig. 2.7). In contrast, the poor performance of the pairs F₅-F_{wt} and F₁₁-F₁₂ was most likely due to the relatively high backgrounds recorded (Fig. 2.7). The differently observed levels of background activity cannot be explained. Nevertheless, a reason for the limited performance of the set F₁₁-F₁₂ at transient RMCE might be also found in the results of the PCR test, which predicted F₁₁ and F₁₂, due to the low recognition potential, as the most non-appropriate FRT pair for RMCE (Fig. 2.3).

To exclude promiscuous recombination between two donor vectors with different sets of FRTs analysis of cross-recombination potential between them was a critical test. Since in the multiplexing-RMCE scenario both donor plasmids exist simultaneously in one cell, homologous or site-specific recombination among them would give rise to exchange or translocation of plasmid-derived DNA stretches. Successful SSR among one FRT site would lead to the merge of both donor plasmids. Except F_5 - F_{wt} , all sets of FRTs added to the standard combination F_3 - F_{wt} showed no significant indication of unwanted cross-recombination. Experiment P: F_3 - F_{wt} **X** Δ P: F_5 - F_{wt} + Flp, however, showed a significant higher transient RMCE level than the other samples of group (x) (Fig. 2.7). As indicated in PCR-tests by Schlake & Bode (1994), besides the anticipated recombination of both F_{wt} -sites, cross-recombination among F_3 and F_5 was likely to happen here, supposedly due to the fact that spacers of both sites differ merely in 2 bp (violation of 4 bp rule).

Taken together, transient RMCE revealed combinations F_{13} - F_{14} and F_{14} - F_{15} to have a satisfying (transient) RMCE potential. Exchange vectors with these sites also showed no indication for promiscuous recombination with a F_3 - F_{wt} tagged plasmid. These data together with the data from the PCR test qualified these sites for further analyses concerning genomic RMCE.

3.3 Genomic RMCE

3.3.1 Sets F_{11} - F_{12} and F_{13} - F_{14} are both capable of genomic RMCE in a polyclonal cell line

RMCE in a genomic background proves to be more laborious than transient RMCE. After genomic integration of a parental construct, selection and single cell deposition has to be applied in order to gain single copies of integrated vectors, which, after characterization by Southern Blot, can be subjected to subsequent RMCE steps. For a final proof of cassette exchange, PCR or Southern Blot analysis are required.

For verification of multiplexing-RMCE chosen candidates F_{11} - F_{12} and F_{13} - F_{14} (both with F_3 - F_{wt}) (ch. 3.4) were subjected to a simple genomic RMCE test. Combination

F₁₄-F₁₅, which gave the best results for transient RMCE was not considered for further experiments due to the late implementation of these sites in the transient RMCE experimental series.

In classic protocols for RMCE (Goetze *et al.*, 2005) transfected cell lines with a single copy vector-tag had to be sorted or positive/negative selection strategies had to be applied to obtain PCR-derived proof for RMCE. Here, two days after a single transfection of a multiple copy vector-tagged cell line with donor plasmids and Flpo-expressing plasmids, signals indicating successful targeting could be PCR-amplified (Fig. 2.9). Similar results were shown by Qiao *et al.*, (2009). Seemingly, the donor plasmids are able to recombine at several parental tags and the likelihood (and hence accessibility) of actively expressing and open loci is higher in a polyclonal than in a monoclonal culture.

For a final proof of RMCE, single cell clones have to be obtained and additional Southern Blot studies are necessary, which is laborious. This method, however, is a quick test to evaluate performance of novel FRT pairs in a genomic environment.

The generation of a RMCE⁺-PCR signal after transfection of both polyclonal cultures (F₁₁-tkneo-F₁₂+220 or F₁₃-tkneo-F₁₄+220) indicated the feasibility of both novel FRT pairs in a genomic context of NIH/3T3 cells. Anyhow, signal intensities for both RMCE⁺ PCR bands were clearly different. While in the F₁₁-tkneo-F₁₂+220 polyclonal culture an intense signal indicating RMCE was seen, just a weak one appeared in case of F₁₃-tkneo-F₁₄+220 (Fig. 2.9). In contrast to transient RMCE and the PCR test suggesting F₁₃-F₁₄ as the superior of both pairs, here, the opposite seems to be true. Reasons for that might be the average three-fold reduced transfection efficiency for F₁₃-F₁₄-tagged polyclonal culture of donors and Flpo-puro (F₁₁-F₁₂: 37% versus F₁₃-F₁₄: 12%). The three-fold lower amount of transfected donor DNA and Flpo-puro in the F₁₃-F₁₄-tagged culture might severely reduce RMCE efficiency. In a previous study it was shown that high doses of applied recombinase caused completion of the recombination process within a few hours (Peitz *et al.* 2002), and that Flp-dose is a significant parameter for cassette exchange efficiency in ES cells (Roebroek *et al.*, 2006). Another explanation could be a difference in the genomic accessibility of integrated parental vectors between the F₁₃-F₁₄-tagged polyclonal culture compared to the F₁₁-F₁₂-tagged polyclonal culture. Of note, position effects can decrease

efficiencies for site specific recombination between genomically residing target sites (Vooijs *et al.*, 2001), which might also be true here. However, although these experiments had suggested the combination F_{13} - F_{14} to be less effective as F_{11} - F_{12} , its potential as a second pair for final multiplexing-RMCE experiments was investigated.

3.4 Multiplexing-RMCE

3.4.1 Transfection and sorting steps are limiting factors for multiplexing-RMCE

F_{11} - F_{12} - and F_{13} - F_{14} -based parental targeting constructs were electroporated into clone N1 to generate multiplexing-RMCE competent cell lines. N1 harbors a single copy of F_3 -hygk- F_{wt} (Seibler J., Dissertation, TU-Braunschweig, 1999). The choice of N1 goes back to studies by Goetze and colleagues (2005), who showed that N1 in contrast to four other evaluated clones is a long-term high expresser of the transgene (Fig. 2.10). This locus was concluded not to be subject to epigenetic effects provoking heterochromatization and silencing.

After modification of N1 there was a need to recover clones with a single vector copy from pools of M (target 1: F_3 -hygk- F_{wt} // target 2: F_{11} -tkneo- $F_{12}+220$) and O (target 1: F_3 -hygk- F_{wt} // target 2: F_{13} -tkneo- $F_{14}+220$). A cell line with two vector copy integrations would increase chances of intramolecular recombination among homologous FRT sites of parental vectors, for instance F_{11} with F_{11} or F_{12} with F_{12} in the case of two integrated copies of F_{11} -tkneo- $F_{12}+220$. In the presence of active Flp-recombinase this would lead to excision or translocation of chromosomal DNA stretches and promote chromosomal instability (Bode *et al.*, 2000b).

Single copy integration of novel parental vectors was verified by Southern Blot analyses for several clones (Fig. 2.11). Clones O5 and M28 were used for the ultimate multiplexing-RMCE series. These experiments were initiated by a single transfection applying both donors (each 1.5 μ g) and Flpo-puro plasmids (1.0 μ g). Compared to the standard protocol, a difference of this strategy was the reduced amount of applied donor plasmids (1.5 μ g) compared to the double amount (3.0 μ g) used in

previous studies for mammalian cell lines (Qiao *et al.*, 2009). This became necessary, because Metafectene[®] transfection of more than 4 µg plasmid DNA per 1.5E5 NIH/3T3 cells has severe effects on cell viability (data not shown).

Transfection efficiencies prior to the first FACS sorting step did not exceed 30 % and contained a high fraction of cells, which were non-fluorescent or showed just d2eGFP or RFPex expression but no coexpression of both markers (data not shown). Only a few hundred cells of the initially 6.0E6 cells seeded for one experiment were hit by both expressing donor plasmids as indicated by double fluorescence. The low cotransfection rates are ascribed to impaired transfection efficiencies using Metafectene[®], even though these were approximately 30% if only one donor was transfected (data not shown). Other transfection reagents were not tested in this context. Electroporation is used as a method for delivery of expression vectors but it also can harm integrity of genomic DNA by random introduction of nicks or double strand breaks (Meaking *et al.*, 1995). It seems obvious that exogenously applied circular, supercoiled DNA appears to be prone for DNA damage to the same extent as reported for genomic DNA, which makes this delivery method non-appropriate for RMCE. However, electroporation is still a method of choice for cassette exchange studies of ES-cells (Schebelle *et al.*, 2010). One reason for this might be the possibility to deliver much more plasmid DNA than with lipofection.

In order to achieve a higher transfection rate, an alternative strategy would be sequential targeting of both loci. Using Metafectene[®], this would permit application of a higher amount of donor DNA (3.0 µg) for each locus, which would give rise to higher efficiencies for transfection and sorting. This approach, however, would suffer from the need to find one locus-exchanged master clones. This would mean much more efforts as two steps of transfection, FACS sorting, single cell deposition and negative selection would be inevitable.

3.4.2 Targeting both loci of O5 is feasible

After double sorting of clone O5' transfected cells and prior negative selection, first indications for RMCE of both target loci could be derived from PCR analyses (Fig. 2.12b). PCR-amplified signals indicating exchange at locus F₁₃-tkneo-F₁₄+220 (locus

F_{13} - F_{14}) were faint compared to the intense signal for the locus F_3 -hygtk- F_{wt} (locus F_3 - F_{wt}) (Fig. 2.12b). The difference in the PCR-derived signal strength might be ascribed to the fact that the previously characterized locus F_3 - F_{wt} is more accessible and hence more favored for recombination than the locus F_{13} - F_{14} , as indicated by Goetze *et al.*, (2005) and described in ch. 1.3.2.

3.4.2.1 **Negative selection of targeted clone O5 with Ganciclovir is dose-dependent**

Enrichment of O5-derived cells, whose both loci were exchanged, became feasible by the application of negative selection drug Ganciclovir. Both target plasmids contain a thymidine kinase (tk) marker in the parental tag, which are getting removed by RMCE. After negative selection, Flp^+ -samples showed bands for targeted integration at both loci with comparable density in the PCR analyses (cf. Fig. 2.12b with Fig. 2.12c). In former experiments by Qiao *et al.*, (2009), negative selection with Ganciclovir was carried out in CHO cells at a concentration of 3000 nM. Here, selection with this concentration gave rise to no or a very low number of growing colonies (Tab. 2.3). By adjusting the concentration of Ganc to 30-fold lower level resistant colonies grew in the Flp^+ 2x sorted samples. This suggests that Ganc concentrations of 3000 and 1000 nM are lethal for Flp^+ 2x sorted samples, whereas the concentration of 100 nM is not. It might be necessary to clarify why Ganciclovir shows a dose-dependant toxic effect on all cells of a population, in which a fraction has no residual thymidine kinase activity due to a shown double exchange (Fig 2.12 c). Studies by Bi *et al.*, (1993) indicated that tk expressing cells (tk^+ -cells) (here, non-exchanged cells of O5) can mediate cell killing on tk non-expressing cells (tk^- -cells) (here, doubly exchanged cells of O5) by application of Ganc. Prerequisites for this bystander effect are direct cell-cell contacts, which were avoided in this setup by low density cell seeding (2E4 cells per 10 cm culture dish). It is unclear if the bystander effect is dose-dependent, and if elevated concentrations of Ganc can escalate the toxic impact of the deoxyguanosine triphosphate (dGTP) analogue also to tk^- -cells, which are not in cell-cell contact with tk^+ -cells.

3.4.3 PCR as well as Southern blot analyses prove double exchange in subclones of O5

Single cell deposition via limiting dilution was applied to isolate cells from the Ganc-resistant culture of O5, which was positively tested for both exchange reactions (Fig. 2.12c). Single clone analysis for exchange by PCR and Southern blot was meant to demonstrate multiplexing-RMCE in one cell. 29 tested clones by PCR and 8 clones by Southern Blot showed evidence for a double exchange (Fig. 2.13-2.15). Exchange frequency for PCR was determined to be 100% for subclones of O5 while Southern Blot revealed exchange efficiency of 89% for nine tested subclones. In all RMCE-positive samples investigated by Southern Blot no randomly integrated donor plasmids were seen (Fig. 2.15).

Analysis of clone 19 revealed a band not matching random integration or targeted integration. There are two explanations that interpret this phenomenon, though they are mutually exclusive. The signal might originate from a merge reaction between both types of transfected donor plasmids. Due to the presence of prokaryotic plasmid parts the larger backbone stretches of homology among the donors F_3 -d2egfp- F_{wt} and F_{13} -rfpex- F_{14} or, alternatively, a cross-recombination between two heterospecific FRTs might have led to a recombination-mediated fusion of donors. RMCE⁺-PCR data for both loci contradict this observation. Taking this into account, another explanation might be the imperfect recombination among donor and parental vector sequence, which would yield an intermediate product (Schlake & Bode, 1994). This intermediate situation would allow positive PCR results for RMCE, since current PCR strategy evaluates cassette exchange at 5'-FRT but not at both sides. Yet, extraordinary bands in the Southern Blot of clone 19 appear for both loci, which would imply the illegitimate interaction between the intermediates, which might simultaneously arise at both loci. Although such a side-reaction is a very rare phenomenon, it would be safely excluded if synchronous RMCE at both loci would be replaced by successive RMCE steps.

3.4.4 Targeting both loci of M28 is not feasible

The application of this protocol to clone M28 yielded signals for exchange at locus F_3 - F_{wt} but not at locus F_{11} - F_{12} before and after application of negative selection (data not shown). Spontaneous resistance of the non-targeted locus F_{11} - F_{12} of M28 could explain this phenomenon, since O5 did not bear any resistant clones in the Flp^- samples (Tab. 2.3). Ganc-derived spontaneous resistance was reported previously (Seibler *et al.*, 1998). One reason might be the low dose level of Ganciclovir applied (100 nM/mL), enhancing chances of spontaneously resistant clones to form.

Spontaneous resistance might be the consequence of genomic silencing. Vooijs *et al.*, (2001) demonstrated position effects for genomic site-specific recombination recognition sites. In the present case, similar effects might cause silencing of locus M28. A more likely explanation, however, is the Flp -mediated excision of the parental vectors' *tkneo* fusion gene (Oumard *et al.*, 2006). This can be ascribed to residual cross-recombination between F_{11} and F_{12} in M28. This effect was also indicated in a PCR test, which compared F_{11} with F_{12} (Turan, S., diploma thesis, TU-Braunschweig, 2007). Such an effect could be prevented by applying sites F_{11} and F_{12} in inverse orientation (in which case the selection gene would not be excised).

In contrast to combination F_{13} - F_{14} , the FRT pair F_{11} - F_{12} appears to have an inferior potential supporting RMCE. This explanation is also strengthened by the fact that in RMCE studies with single copy clones of parental tag F_3 -hyg^{tk}- F_{wt} six out of six derived cell lines were successfully analyzed for authentic exchange (Seibler J., Dissertation, TU-Braunschweig, 1999).

3.5 Cre and Flp in the context of (multiplexing)-RMCE

Since Sauer (1996), proposed a multiple targeting system of naturally occurring loxP pseudo sites, which resembles multiplexed integration of donor vectors, no reports describing attempts in that direction were published. However, a functional multiple targeting system for Flp -RMCE could be presented in this work. The question why

the Cre/loxP system in contrast to the Flp/FRT system is presumably not capable for multiplexing-RMCE still has to be addressed. Nevertheless, there are certain differences among both systems, for instance the enzymatic mechanisms.

While both, Cre and Flp recombinases, are members of the λ integrase family of tyrosine recombinases and while both show similarities in the outcome of their reaction mechanism, regarding integrating, excising or inverting DNA, their recognition sites are different (for FRT, the inverted repeats surrounding the 8 bp spacer are complemented by a third 13 bp repeat with potential regulatory function; Fig.2.1). There is a number of indications that the mechanism of recombination is related but definitely not identical (Sadowski, 1993; Kilby *et al.*, 1993; chapter 1.3.1).

3.5.1 Comparison of mutant recognition sites indicates enhanced promiscuity and a lower self-recognition potential of lox sites

Cre-RMCE was first shown with a non-inverted pair of heterospecific loxP sites. The mutant lox511 (Hoess *et al.*, 1986) and the loxP_{wt} site were shown to be functional for RMCE in NIH/3T3 cells by Bethke & Sauer, (1997). In mouse erythroleukemia cells (Bouhassira *et al.* 1997) and ES cells (Feng *et al.*, 1999) Cre-RMCE was reported with the same combination of lox sites but inversely oriented. Further targeting could be shown on fertilized mouse oocytes (Lauth *et al.*, 2000).

However, promiscuous recombination was shown among loxP_{wt} and the heterospecific lox511 mutant, whose spacer has been changed in one position compared to the loxP_{wt} spacer. As this was demonstrated *in vitro* (Lee *et al.*, 1998), in bacteria (Siegel *et al.*, 2001), cell lines and mouse oocytes (Lauth *et al.*, 2000), it had to be concluded that lox511 is not appropriate as a candidate site for Cre-RMCE (Kolb *et al.*, 2001; Langer *et al.*, 2002). Novel lox mutants such as lox2272 and lox5171 (Lee & Saito, 1998) (spacer changed in 2 positions), lox257 (Wong *et al.*, 2005) (spacer changed in 3 positions) and m2 (Langer *et al.*, 2002) (spacer changed in 6 positions) were developed in mutagenesis screens. Improvement in terms of incompatibility with loxP_{wt} was given for lox2272, yet cross-recombination was not completely abolished (Kolb *et al.*, 2001). Even more seriously, Mlynárová and colleagues (2002) detected increased cross-recombination of lox511 or lox2272 mutants with loxP_{wt} site, if their

recognition sequence was close to sequences with palindromic character. In a high throughput screening approach comparing many mutant lox sites among each other Missirlis *et al.*, (2006) had to conclude that self-recognition among identical lox sites was at most marginally preferred over recombination among heterospecific lox sites. Strategies to avoid crossinteraction among the RT sites were developed, and made use of parallel application of Flp and Cre-recombinase to perform a combined Cre/Flp RMCE. Here, the combination of the F_{wt} site and a loxP wild-type were used for RMCE in ES cells (Lauth *et al.*, 2002).

Prerequisites for a Cre-multiplexing approach would be to obtain lox mutants, which show no crossinteraction among each other. However, Jung *et al.*, 2007, showed significant cross-recombination among five mutant lox sites (lox2272, m2, m3, m7, m11), even though this mutants showed no cross-recombination with the loxP_{wt} site. This work exemplifies the difficulties in finding potential lox mutants for Cre-multiplexing RMCE

For Flp-RMCE, however, the use of conventional mutant FRTs F_3 and F_5 did not exhibit any cross-recombination with F_{wt} in PCR tests (Schlake & Bode, 1994; this work). Also in many studies concerning RMCE these sites were never found to recombine with F_{wt} site in cell lines (Goetze *et al.* 2005; Qiao *et al.* 2009) and ES cells (Schübeler *et al.* 1998; Lauth *et al.* 2002; Cobellis *et al.* 2005). Similarly, our novel FRTs did never show indication for promiscuous recombination with the F_{wt} site in critical PCR tests (Fig. 2.4).

Next to analysis of crossinteraction, evaluation of self-recognition capacity between mutant lox sites was shown to be contradictory. Siegel *et al.*, 2001, could show that mutants lox511 and lox2272 have a self-recognition potential being similar to the recognition potential between two loxP_{wt} sites. In a later study mutant m2 was reported to have similar activities as the loxP_{wt} site as well (Langer *et al.*, 2002). However, Lee and Saito, (1998), illustrated a reduced self-recognition potential among homospecific 1 bp spacer mutant lox sites (for instance lox511). A 2 bp mutation of the lox spacer further decreased the self-recognition potential. Among 15 of 30 double mutants tested, recombination potential was below 10% of the wild type standard. Incomplete recombination of lox511 sites was also seen in a Cre-RMCE study done with a loxP_{wt} and lox511 site pair (Lauth *et al.*, 2000). In contrast, self-recognition capacity of most

mutant FRT sites was comparable to the self-recognition potential of the F_{wt} site (Schlake & Bode, 1994; Fig. 2.3).

The question why self-recognition between homospecific lox mutant sites seems to be more impaired than self-recognition between FRT-mutant sites still needs to be addressed. One model suggests that shown differences in the spacers' cleavage-site between Cre and Flp have a more critical effect on recombination efficiencies among two mutated lox sites than among two mutated FRT sites. Of note, Flp cuts at the 5'-margin base pair at position +4/-4 (Fig. 2.1), whereas Cre cuts at analogous base pair +3/-3 of its spacer.

To conclude, there are several reports for the Cre/lox system showing cross-recombination between mutant lox and loxP_{wt} sites. Two reports argued mutant lox sites to bear equal self-recognition potential like the wild type site. However, two studies claim that the lox mutants have a general decreased self-recognition potential. FRT-mutants indicated non-reactivity versus F_{wt} and other heterospecific sites. There are no indications whatsoever for cross-recombination of four bp spacer mutants such as F_3 and F_5 , nor was there a visibly reduced self-recognition potential. These differences between the Flp/FRT and Cre/loxP system imply the Cre-RMCE to be inferior to Flp-RMCE, which could be a reason for the decreasing rate of applications for Cre-RMCE.

3.5.2 loxP pseudo sites in various genomes interfere with multiplexing-Cre-RMCE

It was reported that, instead of inversion, deletion of DNA, which is flanked by inversely oriented loxP_{wt} sites takes place in *E.coli*, if Cre is overexpressed (Aranda *et al.*, 2001). Further reports in mammalian cells also implied the necessity for dose-limitation of Cre, due to several cases of genotoxicity at high concentrations of Cre (Loonstra *et al.*, 2001; Higashi *et al.*, 2009; Jae Huh *et al.*, 2010,). These limitations were never shown to be essential for the Flp/FRT system (Roebroek *et al.*, 2006; Seibler *et al.*, 1998, Schebelle *et al.*, 2010) in which treated cells were inert even in presence of high doses of Flpe (70 - 100 µg). In contrary, high doses are a proven tool for accelerating the reaction of site specific recombination (Peitz *et al.* 2002) and

thereby necessary to suppress side reactions due to the extended persistence of intermediates. The development of the mouse codon-optimized Flp (Flpo) (Raymond *et al.*, 2007) further advanced the activity level of Flp-recombinase, which is reported to be similar to Cre (Kranz *et al.*, 2010).

Indications for unspecific activity of Cre were the detection of loxP pseudo sites in the mammalian genome (Thyagarajan *et al.*, 2000; Semprini *et al.*, 2007). loxP pseudo sites are putatively responsible for an increased genotoxicity of Cre, due to observation of chromosomal aberrations in mice spermatides after expression of recombinant Cre (Schmidt *et al.*, 2000). There are indications that these sites can get cleaved and that they recombine among each other if Cre is expressed for a prolonged period in the cells. Thyagarajan and colleagues, 2000, could isolate and clone diverse loxP pseudo sites on plasmids and show that these sites get cleaved by Cre. Furthermore, Cre-mediated recombination among pseudo sites was also seen. By comparison, FRT pseudo sites never were detected for the FRT/Flp system thus far suggesting Flp activity to be more specific than Cre activity.

Unspecific activity of Cre might contribute to reported genotoxicity (Jae Huh *et al.*, 2010). Star activity might also originate from the mechanisms of recombination on a molecular level, which are not identical between Flp and Cre. Both systems share an identical organization of their recognition site being composed of two inverted 13 bp repeats surrounding an 8 bp asymmetrical spacer but only FRT sites include an extra repeat b (Fig. 2.1). This repeat might sequester redundant Flp-monomers (protomers) and contribute to a balanced activity of Flp. After binding of the recombinases on the repeats of the recognition target site the process of recombination starts in both systems with the activation of the scissile phosphodiester bond by the catalytic core triad Arg-His-Arg. The active site tyrosine is responsible for the nucleophilic attack in both systems. The cleavage step, however, seems to differ between them. Cre was shown to perform a *trans*-cleavage (Shaikh & Sadowski, 1997), whereas crystal structure studies clearly revealed a *cis*-cleavage step (Guo *et al.*, 1997). The latter described mechanism implies provision of the Arg-His-Arg triad and the site active tyrosine from one bound Cre-protomer. Remarkably, Flp mediates a “*trans*-horizontal cleavage”-step in which the Arg-His-Arg triad and the active tyrosine are provided by two different Flp-protomers (Lee *et al.*, 1999; ch. 1.3.3). Both involved protomers bind to opposite faces of the inverted repeats of a FRT. The

observed non-promiscuous activity of Flp, implying low genotoxicity, might in contrast to Cre be explained by this more demanding cleavage step.

In summary, there are several intriguing facts that disqualify the Cre/loxP system for use in a multiplexing-RMCE system.

- Promiscuous recombination between loxP_{wt} and lox mutant sites: novel mutants like lox2272 and m2 appear to show negligible low cross-recombination with loxP_{wt}, but they showed significant rates of cross-recombination, if compared versus each other in bacteria (Jung et al., 2007). It would be necessary to develop novel heterospecific mutant lox sites and initiate extended cross-recombination studies for Cre-multiplexing-RMCE in mammalian cell lines.
- A shown impaired self-recognition potential of lox mutants compared to mutant FRT sites (ch. 3.5.1).
- Increased chances for aberration, inversion and translocation of chromosomes due to the canonical presence of genomic loxP pseudo sites in mammalian genomes, which severely inhibit the use of expanded targeting technologies.
- Limited use of recombinant Cre-recombinase, due to dose-dependant genotoxicity, which is accompanied by increased cross-recombination among lox-mutants.

3.6 Perspectives

3.6.1 Enhancing efficiency of multiplexing-RMCE

FRT sites

In this study it was shown that RMCE of two donor cassettes targeting two distinct loci with four different heterospecific FRTs is feasible. Since preliminary tests also indicated a good potential for a site combination F₁₄-F₁₅, one of the next tasks would be the usage of this pair together with F₃-F_{wt} and compare the performance of this quadruplet with the F₁₃-F₁₄ applied here.

Flp/donor ratio

There is a persistent need for the optimization of several parameters. As described above (ch. 3.5.2), several studies used far more Flp-encoding vector DNA per cell and could enhance the efficiency of RMCE in ES cells (Tab. 3.2). During genomic RMCE, Flp-recombinase and donor DNA are transiently present in the target cell. This implies sequestration of Flp-protomers by the FRT-sites of the donor DNA (and transient RMCE among the identical donors), which might decrease the amount of free available Flp-protomers for genomic RMCE. To meet this, the amount of Flp-encoding plasmids should be significantly higher than the amount of plasmid DNA:

Tab. 3.2 Comparison of Flp-vector/ donor vector ratios and RMCE efficiencies of several studies

Flp-encoding vector/ donor vector ratio was between 2.3 and 3.4 in studies conducted with murine ES cells. Efficiencies of RMCE ranged between 20-60 %. There are several indications that a ratio below 1 results in a less effective RMCE.

	Cell type	Flp-vector [μg/5E6 cells] (1)	Donor vector [μg/5E6 cells(2)]	Ratio (1)/(2)	RMCE efficiency
Seibler <i>et al.</i> , (1998)	murine ES	170 (Flpe)	50	3.4	21-38%
Roebroek <i>et al.</i> , (2006)	murine ES	100 (Flpe)	30	3.3	38-61%
Schebelle <i>et al.</i> , (2010)	murine ES	35 (Flpo)	15	2.3	40%
Qiao <i>et al.</i> , (2009)	CHO	40 (Flpo)	120	0.3	10%
This work	NIH/3T3	25 (Flpo)	2x40	0.31	ch. 3.4.3

Flp-delivery

For Flp-delivery, novel transduction methods were established, which are more efficient than simple transfection of Flp-encoding vectors. Voelkel *et al.*, 2010, describe the feasibility of transduction of Flp fusion protein particles, which are embedded in a retroviral environment for more efficient delivery of Flp-recombinase. Besides that, Flp-recombinase fused with cell penetrating peptides like TAT (twin-arginine translocation) was used for efficient Flp-transduction of fibroblast cell lines

(Patsch *et al.*, 2010). These novel delivery techniques might be helpful to enhance multiplexing RMCE.

Donor delivery

The provision of the donor plasmid via integration-defective lentiviruses can facilitate the delivery to problematic cell types (Torres *et al.*, 2010, submitted). In order to enhance targeting, the construction of a multiplex donor, harboring both exchange cassette units in one vector could be beneficial regarding several parameters. Since 1.5 µg of DNA was used for cotransfection of each of both donors in NIH/3T3 cells with Metafectene[®], twice this amount for one donor would increase the chance for exchange in a given cell.

Cis-acting elements

Scaffold/matrix attachment regions (S/MAR) are AT-rich sequences, which are associated with the protein backbone of the nucleus. Several reports showed augmentation of transgene expression by application of S/MARs as flanking elements of transgenes (Zahn-Zabal *et al.*, 2001; Goetze *et al.*, 2005). S/MARs structure and remodel chromatin domains and promote transcriptional activity (Bode *et al.*, 2000a). In addition, they act as insulator elements and can block DNA methylation, which is known to cause silencing on genomically integrated transgenes (Goetze *et al.*, 2005; Klehr *et al.*, 1991). In the context of RMCE higher expression level and a higher efficiency of cassette exchange were observed in S/MAR-flanked clones compared to non-S/MAR-flanked clones. This was ascribed to the insulating function and the strand separation potential of the S/MAR elements (Qiao, J. Dissertation, TU-Braunschweig, 2009).

In the context of this work, this system would be feasible for enhancing RMCE efficiency and expression parameters of single or both parental tags.

3.6.2 Combined “polyclonal targeting” and “promoter-outside” strategies simplify characterization of clones and prevent random integration of donors

For a more meaningful determination of exchange efficiencies (ch. 3.4.3) at least ten clones with a unique single copy integration pattern of both parental tags should be applied to multiplexing-RMCE. One way to obtain these clones is described in this work (ch. 2.3.2). However, to ease this effort, a polyclonal pool of $\text{hyg}^r/\text{neo}^r$ cells harboring two parental vectors, each one tagged with an individual pair of heterospecific FRT sites could be subjected to multiplexing-RMCE in one parallel or two successive steps. After sorting of doubly hit cells, targeted clones should survive and grow in the presence of Ganciclovir. Since the likelihood of targeting two loci in a single step is rather low (ch. 3.4.1), the negative selection principle should largely enrich cells with a limited number of genomic addresses. The benefit of this method is the simplicity to obtain clones with a unique integration pattern and a defined expression level without any laborious pre-characterization of single clones.

A related concept was successfully applied in an analogous way for single locus F_3 - F_{wt} RMCE by Qiao *et al.*, 2009. In order to find a high level expresser clone for biotechnological purposes like protein (antibody chain) expression in the Chinese hamster ovary (CHO) mammalian cell line 24 single clones were analyzed for RMCE after targeting of a F_3 - hyg^r - F_{wt} polyclonal pool. These clones showed diverse genomic integration and expression patterns. In order to overcome the frequently reported random integration of donors (Qiao *et al.*, 2009; Seibler J., Dissertation, TU-Braunschweig, 1999) a “promoter-outside” strategy was applied:

A donor, harboring a promoterless F_3 - egfp - F_{wt} exchange cassette was swapped into a SV40- F_3 - hyg^r - F_{wt} tagged parental locus with a promoter lying 5' to the FRT-flanked exchange cassette. Only upon exchange eGFP expression was activated. Cells activated by RMCE could be detected and sorted by FACS. Qiao *et al.*, (2009) could show that just 1 out of 14 analyzed subclones revealed a randomly integrated donor plasmid. The promoter-outside approach appears promising for implementation in future multiplexing-RMCE experiments.

3.6.3 RMCE with several heterospecific sites

Since novel FRT sites indicated satisfying performance, another task would be the construction of large gene domains, in which each sub-area of a domain would be specifically targetable by RMCE. For instance, the independent exchange of promoter units and/or the adjacent GOI *in vivo* would be feasible by RMCE in which each parental tag and each donor is provided with a unique combination of heterospecific FRT sites. According to figure 3.1, a set F-F' flanks a control element such as a promoter or an insulator region and the combination F'-F'' flanks a GOI.

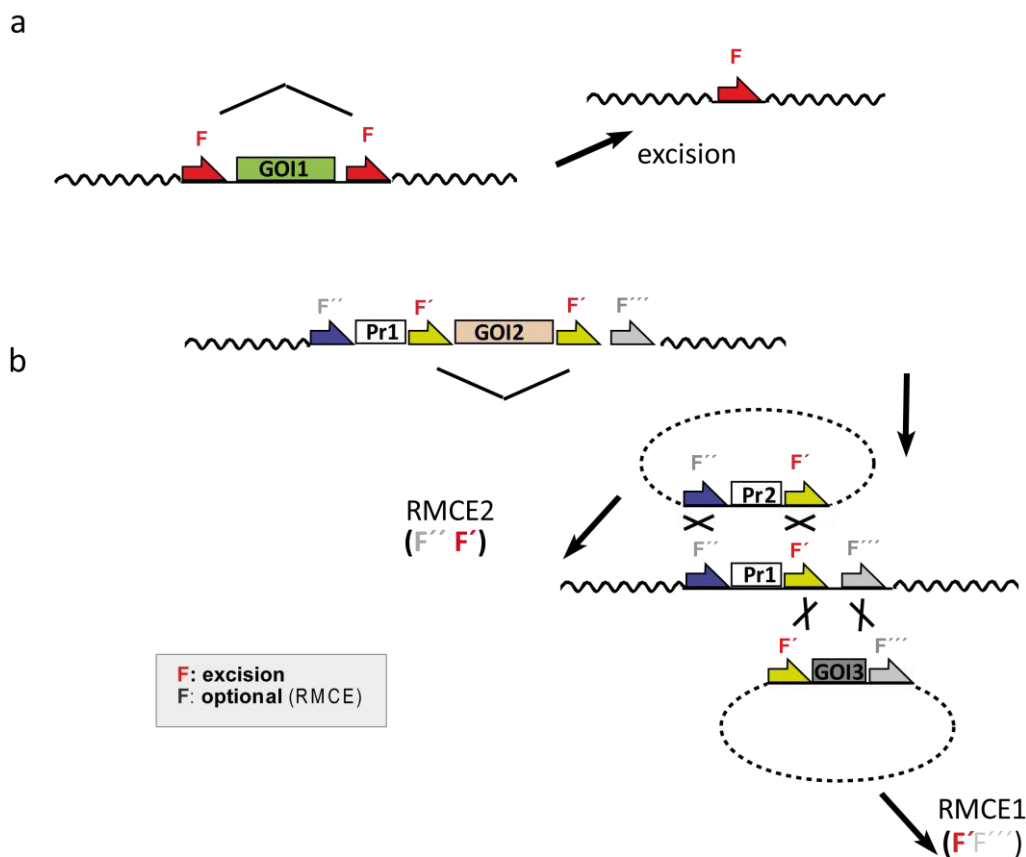


Fig 3.1 Several heterospecific FRTs open various options for a given cellular system

a: flanked allele, GOI1, can be deleted by application of Flp. **b:** combination of excision and RMCE with three heterospecific FRTs: Promoter element (Pr1) is flanked by a heterospecific FRTs (F''-F') while GOI2 is flanked by a homospecific pair of FRTs (F'-F'). In addition, there is another heterospecific site, F''', in 3'-position to F'. This strategy allows excision of the allele GOI2 and optional integration of the allele GOI3 by RMCE with FRT pair F'-F''' (RMCE1). Exchange of promoter Pr1 by promoter Pr2 is another option with pair F''-F' (RMCE2).

This scenario could be useful for the elucidation of *cis*-dependant effects from promoters, insulators or S/MAR regions (ch. 3.6.1) exerting on adjacent genes. These effects were anticipated in Flp-mediated excision studies (Seibler J., Dissertation, TU-Braunschweig, 1999). Thus far, five non-interacting heterospecific FRTs can be combined, resulting in four targetable sub-areas of a transgenic domain.

While “floxing” is the process of excision, mediated by the Cre/loxP system, “flirting” is the analogue mechanism for the Flp/FRT system. The principle of dual floxing was introduced by Buchberger *et al.*, 2007 and used for the conditional knock out of two genes by Cre-mediated deletion of floxed stop sequences, which triggered expression of short hairpin RNA being responsible for the silencing of target genes. Due to the restricted number of heterospecific mutant FRT sites, multiple flirting at several targets has not been applicable yet.

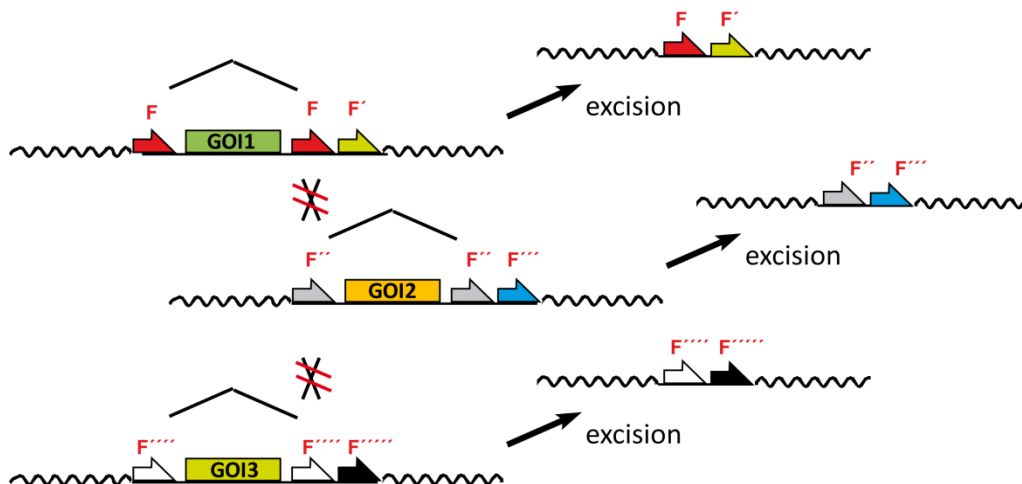


Fig 3.2 multiple flirting and RMCE

Each of several introduced transgenic GOIs is flanked by a different set of homospecific FRTs. After application of Flp, multiple excisions of the GOIs and no subsequential chromosomal rearrangements occur. Optionally, a heterospecific FRT, which is put next to a 3' flanking FRT provides a RMCE-enabling locus. This can be targeted after excision for further modification of the engineered locus.

After excision, remaining FRTs held the risk to undergo subsequent interactions among each other leading to chromosomal translocations. Multiple excisions of

distinct loci are imaginable by applying novel heterospecific FRTs and can be combined to further elaborate the locus via RMCE (Fig. 3.2).

3.6.4 European Conditional Mouse Mutagenesis (EUCOMM) program: generation of targetable pseudo homozygous mice

One aim of the EUCOMM program (www.eucomm.org) is the generation of targetable pseudo homozygous mice. Therefore, an ES cell library has to be established, which allows modification of both alleles of a given gene. A prerequisite of this strategy is that both alleles of a bi-allelic gene have to be knocked out by homologous recombination (ch. 1.4.1) or gene trapping methods (Friedrich & Soriano, 1991; ch. 1.4.4). ES cells, which bear the knocked out genotype, are selected and can be subsequently engineered by the knock-in of wild type alleles, which are flanked by homospecific or heterospecific FRTs (Fig. 3.3).

First allele (allele 1) can be provided with F-F flanks in order to generate a conditional deletion allele. The second allele (allele 2) can be provided with a pair of heterospecific FRTs (F'-F'') to enable targeting of this allele by RMCE (Fig. 3.3). This setup offers the evaluation of gene-dose effects by Flp-mediated deletion of allele 1. Allele 2 can subsequently be exchanged by several variants of mutants (Roebroek *et al.*, 2006), whose phenotypes, for instance, become apparent only in (pseudo-) homozygous mice.

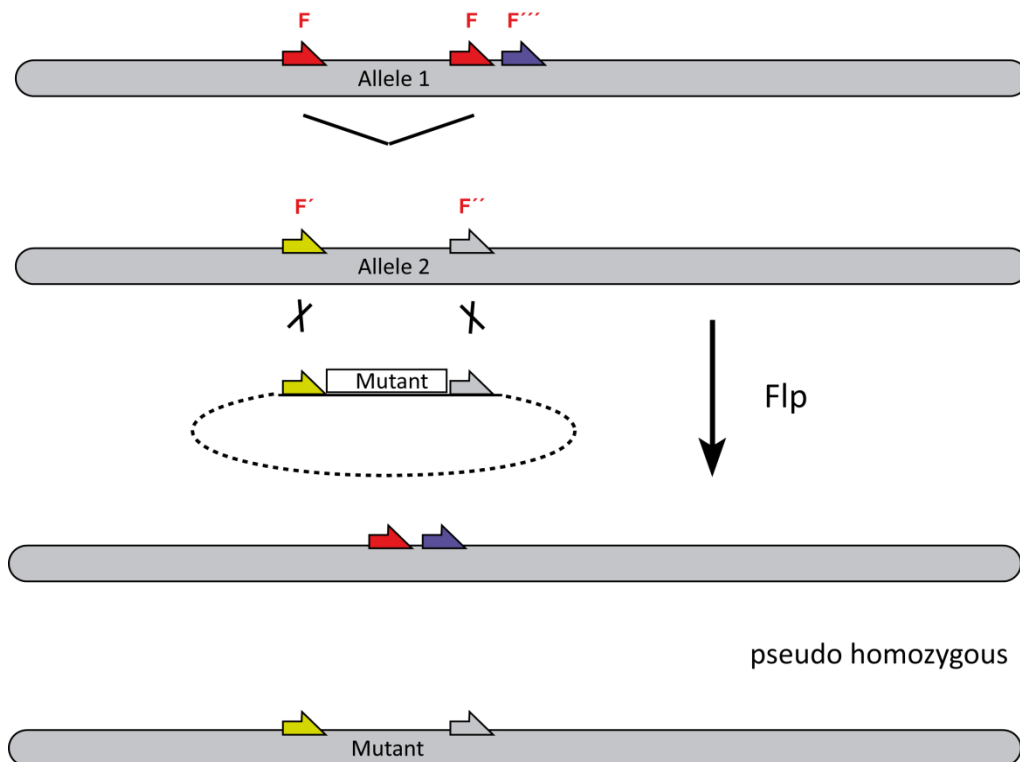


Fig 3.3 Generation of pseudo homozygous mice with several heterospecific FRTs

For bi-allelic genes, heterozygous mice carrying one knock-in modification have to be intercrossed to obtain homozygous offspring. A convenient alternative is pictured here. Prerequisites of this strategy are a previously established knock out of allele 1 and allele 2 by homologous recombination (HR) (not shown). Subsequently, a HR-mediated knock-in introduces the wild type sequence of allele 1 and allele 2 being both flanked by FRTs. Here, one allele is flanked by wild type FRT sites (allele 1), and the second allele is flanked by heterospecific FRTs (allele 2). Flp-mediated excision of allele 1 and modification of allele 2 by RMCE give rise to a mutant pseudo homozygous phenotype. Optionally, integration of a second heterospecific site (F''') at allele 1 enables RMCE at that locus.

For further modification, allele 1 could be also provided with yet another incompatible FRT site 3' to the flanked allele to form another RMCE-compatible site. This strategy would be beneficial for the characterization of genes, in which both alleles bear the same mutation or one allele bears a mutation, whereas the other one remains wild type.

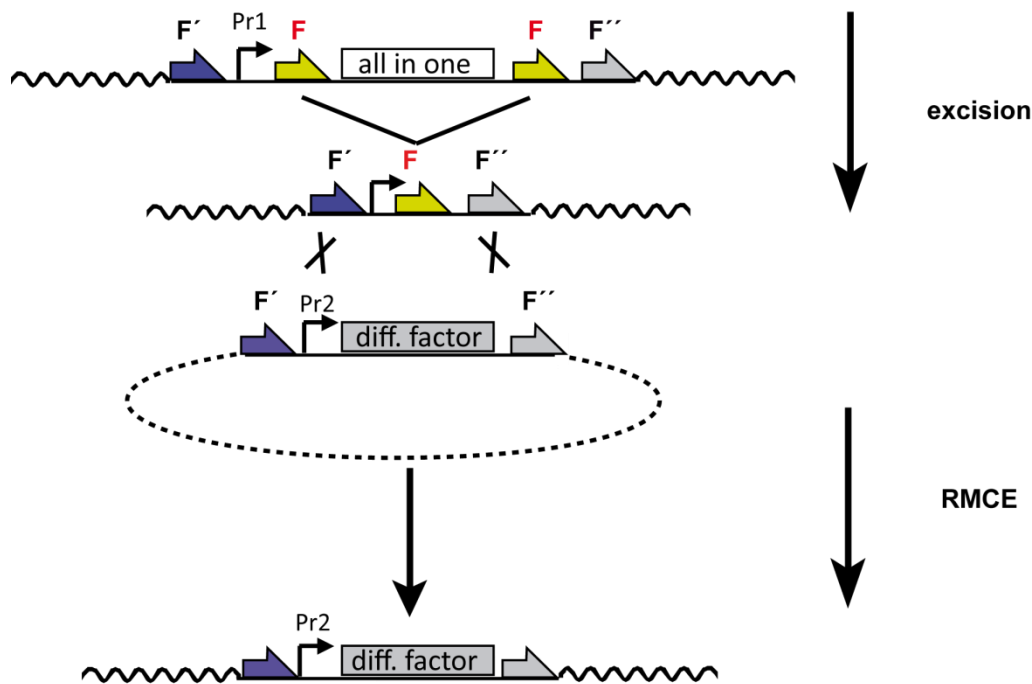


Fig 3.4 Inhibition of teratoma formation and provision of safe harbors for transgenes by flirting and RMCE

Reprogramming of different cell types to pluripotent iPS cells can be triggered by (non-) viral integration and expression of transcription factors *oct4*, *klf4*, *sox2* and *c-myc* referred to as 'all in one'. Withdrawal of the 'all in one' cassette is desirable after the reprogramming process is finished. F_{wt} site flanking the 'all in one' cassette can be excised. Targeted insertion of a differentiation factor can be established by RMCE with two flanking heterospecific FRTs F'-F''. RMCE with set F'-F'' enables differentiation factor(s) to get under control of previously used promoter (not shown). Swapping of the promoter by other promoters is feasible by RMCE with combination F'-F.

3.6.5 (Multiplexing)-RMCE in ES and iPS cells

Currently, a growing number of projects emerge directed at modifying embryonic stem (ES) cells and induced pluripotent stem (iPS) cells with the help of RMCE. iPS can be generated by the retroviral modification of murine embryonic fibroblasts (MEF) by four transcription factors, which play a key role for dedifferentiation (Takahashi *et al.*, 2006). In later studies these four factors could be included in one lentiviral reprogramming cassette (Voelkel *et al.*, 2010). By insertion of the combination of the sites F₃-F_{wt} in the ΔU3 region of the long terminal repeat (LTR), the modified LTR is copied to the 5'-region of the lentiviral cassette after reverse transcription. In this scenario both LTR flank the reprogramming cassette, which now can be engineered by

Flp-RMCE. The expression of the four factors is supposed to be silenced once the re-programming process is finished. Yet, the risk for remaining active cells to initiate teratoma formation remains. To overcome this situation, removal of the re-programming unit by cassette exchange or excision is suggested (Fig. 3.4).

Genomic excision by protein particle delivery of Flpo was already shown by Voelkel *et al.*, (2010). Another strategy would be the random tagging of existing ES or iPS, for instance, by introduction of RMCE-compatible cDNA via the Sleeping Beauty transposase system (Ivics *et al.*, 2009). This would allow modification of active transcriptional spots, which is not assured for the first strategy, described above. For both strategies, RMCE would allow targeted insertion of transcription factors like *hoxb4* into a pre-characterized locus preventing disadvantageous effects like insertional mutagenesis. Ectopic expression of *hoxb4* can trigger differentiation of ES cells (Kyba *et al.*, 2002) and presumably iPS cells into hematopoietic multipotent progenitors.

4 Material & Methods

4.1 Instrumentation, Chemicals, Enzymes and Computer Programs

4.1.1 Instrumentation

Centrifuges :	Eppendorf Centrifuge 5417 C Heraeus-Christ Biofuge pico Heraeus-Christ Biofuge fresco Heraeus pico 17 centrifuge Hettich Rontanta/S
Thermo-centrifuge	Fixed angle rotor: GSA, GS3, SS34 Jouan CR412 Heraeus Multifuge 3SR Sigma 2K-15 Sigma 3K-20 Sorvall Instruments Superspeed RC5-C Swinging cup rotor: HB4
Thermomixer	Eppendorf Thermomixer compact
UV-exposition chamber	Hanau
Gel documentation	Bio-Rad Universal Hood II
Scales	Sartorius CPA64, CP44235
pH-meter	Omnilab seven easy
Fridge	Liebherr Premium
Vortexer	Hobein AG Vortex-Genie™ Hobein AG Vortex-Genie 2™
Photometer	Nanodrop Spectrophotometer ND-100, Peglab
Scintillation Counter	Beckman Counter LS 6500

Electrophoresis Chamber	BRL Horizon 58 BRL Horizon 1114 BRL Horizon 2025
Microscope	Labovet Olympus CK2 Leitz
Fluorescence Microscope	Leica DMIL Zeiss Observer.Z1
Phosphor-Imager	Molecular Dynamics Storm 860
Exposition Chamber	Rego X-ray Exposition Cassette.
FACS Scanner	Becton Dickinson FACSCalibur
FACS Sorter	Beckman Coulter Moflo XDP
Cell Culture Incubator	Labor-Technik-Goettingen Labotect CO ₂ - inkubator C2000 Heraeus BBD 6620
Clean Benches	Mecaplex, Sterilcard Hood VBM600 and SG400 Heraeus, HLB 2448 Heraeus, HSP 18
Thermo-Cycler	Biometra T3 Thermocycler Biometra Tpersonal Thermocycler Biometra Tgradient Thermocycler
Electroporator	Hercules Bio-RAD Gene Pulser and Pulse Controller
Hybridization Incubator	Stuart Scientific Hybridization Oven
Milli-Q Water Purifier	Millipore Milli-Q
Power Supply	Bio-RAD Power PAC 300
Cell Counter	Schaerfe System CASY1
Processor (Developer)	Kodak X-OMAT 1000

4.1.2 Material

Chemicals used in this study were obtained from Amersham, Bayer, BD, Biolabs, Bio-rad, Boehringer, Biozym, Disco, Fluka, Gibco, Merck, Pharmacia, Roche, Roth, Qiagen, Serva and Sigma. Enzymes were purchased from New England Biolabs, Fermentas and Roche.

Oligonucleotides were synthesized by Eurofins MWG Operon. Radioactive chemicals and labeling kits (DecaLabel™) were purchased from MBI Fermentas, Amersham and Hartmann. DNA was sequenced by SeqLab (Sequence Laboratories Göttingen). Plastic materials used in cell culture were bought from Gibco, Greiner, Nunc, Sarstedt, Caromed and TPP.

4.1.3 Computer programs

This manuscript was written with the program Microsoft® Office Word 2007 and Microsoft® Excel 2007. Processing and conversion of images was performed with Microsoft® Office Powerpoint 2007, Adobe Photoshop 6.0 and Adobe Illustrator CS2.

Analysis of DNA sequences, search for sequences, alignment of sequences, restriction sites for endonucleases and simulation of cloning was done with Vector NTI 6.0 (Invitrogen). The processing of FACS analyses data was done with the program Cell Quest Pro (Becton Dickinson) and by FlowJo 7.2.2 software (Tree Star, Inc.). Diagrams were constructed by the program Graph Prism by GraphPad Software. Chromas Version 1.45 (Conor McCarthy, School of Health Science, Griffith University Queensland, Australia) was used for analyzing sequencing results.

4.2 General methods

4.2.1 Sterilizing

All flasks and material of glass were heat-sterilized at 180°C. All solutions were sterile-filtered (0.22 µm pore size) or autoclaved (20 min, 121°C, 1 bar).

4.2.2 Determining concentration of DNA

4.2.2.1 ... photometric

The photometric extinction of a nucleic acid was estimated at 260 nm. The concentration of double stranded DNA is approximately 50µg/µl if the extinction is 1. The ratio of extinction determined at 260 nm versus 280 nm (OD_{260}/OD_{280}) should be at 1.8. The ratio OD_{260} versus OD_{230} (OD_{260}/OD_{230}) should be 2.25.

4.2.2.2 ... electrophoretic

In an agarose gel the intensity of fluorescence visualized by UV-light is proportional to the concentration of DNA marked by ethidium bromide. If the concentration is unknown it can be determined by comparison to a reference DNA sample (reference marker), which concentration is known.

4.3 Use of *Escherichia coli* (E.coli)

4.3.1 Chosen *E.coli* strains

- DH10B: [F' mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 deoR recA1 ara Δ 139 Δ (ara, leu)7697 galU galK λ^- rpsL end A1 nupG λ^- tonA] (Gibco BRL)
- XL1 Blue: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZ Δ M15 Tn10 (Tetr)] (Stratagene)
- Top10F': F'{lacIq Tn10 (Tetr)}, mcrA, Δ (mrr-hsdRMS-mcrBC), Φ 80lacZ Δ M15, Δ lacX74, deoR, recA1, araD139, Δ (ara-leu)7697, galU, galK, rpsL, endA1, nupG; (Invitrogen)

4.3.2 Culture media and antibiotics for bacteria

- | | |
|-------------|-------------------------------------|
| LB medium: | 10 g/l Bacto-Trypton |
| | 5 g/l Bacto-yeast extract |
| | 10 g/l NaCl, autoclaved |
| Ampicillin: | 50mg/ml in ethanol, stored at -20°C |

4.3.3 Preparation of agar plates

15 g of agar per liter were added to the LB medium. For agar plates containing Ampicillin antibiotics, the sterile Ampicillin stock solution (c= 50 mg/ml) was diluted 1/1000 into the medium.

4.3.4 Cultivation of *E.coli*

E.coli was incubated at 37°C in a shaking liquid LB culture ($\pm$ antibiotics). In a pre-culture 5 ml were inoculated with a picked colony for 6 hours. The main culture (50 - 1000 ml) was inoculated with the pre-culture.

4.3.5 Preparation of electro-competent bacteria

1 l of LB medium was inoculated with 5 ml of an overnight culture. The cells grew at 37°C at 180 rpm until the cell suspension had reached an OD₆₀₀ of 0.5 - 1.0. Subsequently, they were centrifuged for 10 min in a cooled GS3-rotor at 3.000 rpm. The cell pellet was re-suspended in 1 l cold water with 10% glycerin and centrifuged as mentioned before. After this, again, the pellet was re-suspended in 1000 ml cold water with 10% glycerin and centrifuged again. The cell pellet was re-suspended in 20 ml cold 10% glycerin (v/v), centrifuged and then re-suspended in 2-3 ml 10% glycerin. This cell suspension was portioned into aliquots (50 μ l), shock frozen into liquid nitrogen and stored at -70°C.

4.3.6 Transformation of DNA into chemo-competent *E.coli*

The chemo-competent bacteria XL1 Blue or Top10F' were thawed on ice for 2 - 5 min. 10 μ l ligation DNA was mixed gently with 100 μ l of bacteria and put back on ice for 10 min. The mixture was put on 42°C for 90 sec and then back on ice for 1 min. 300 μ l ml of LB medium was provided into a 1.5 ml vial and the heat-shocked mixture was added to that and put on a thermomixer (37°C, 500 rpm) shaking for 20 min. The bacteria solution was plated on pre-warmed agar plates with a Drigalski spatula, and the agar plates were put in a 37°C oven overnight.

4.3.7 Conservation of bacterial colonies

For short-term conservation bacteria were plated on agar plates, incubated at 37°C overnight and kept at 4°C. For long-term conservation a 1 ml overnight culture was mixed with 87% glycerin in a 1/1 ratio and was conserved at -70°C.

4.4 Isolation of nucleic acid

4.4.1 Isolation of plasmids in an analytical manner

4.4.1.1 “Boiling Prep” (Holmes and Quigley, 1981)

This method is appropriate for isolation of plasmid DNA for several *E.coli* strains

STET	50 mM	EDTA
	10 mM	Tris (pH 8)
	8%	Sucrose
	0.5%	Triton
LTE	10 mg	Lysozyme/ 1mL TE
	7.5 M	NH ₄ Ac
TER	10 µg	RNase A/ 1ml TE

2 ml of an *E.coli* LB-culture grown under presence of Ampicillin was shaken at 37°C (180 rpm) in test tubes for approximately 16 hours. Suspensions were put into 2.2 ml vials and were centrifuged (1 min, 5.000 rpm). The pellets were resuspended in 500 µl of STET buffer and provided with 50 µl LTE for 2-3 min at room temperature. After that, the samples were incubated for 90 sec in an Eppendorf Thermomixer at 95°C. The samples were centrifuged (5 min, 13.000 rpm). The viscous pellet was removed with a yellow pipette tip. 50 µl of 7.5 M NH₄Ac and 500 µl Isopropanol were added. After vortexing (10 sec) and centrifuging (5 min, 13.000 rpm) the supernatant was discarded and the pellet was dissolved with 50 µl TER solution.

4.4.1.2 Plasmid DNA isolation with QIAprep Spin Miniprep Kit

Pelleted bacterial cells were treated with 250 µl of buffer P1 (see below) and transferred to a 1.5 ml vial. 250 µl of buffer P2 (see below) were added for lysis of bacteria and inverted for 1 min. 350 µl of N3 were added and immediately inverted for 1 min. The 1.5 ml vials were centrifuged (10 min, 13.000 rpm, 4°C). The supernatant was applied to the QIAprep spin column and centrifuged for 60 sec at 13.000 rpm. The flow-through was discarded and the column was washed with 750 µl PE buffer. After centrifugation (1 min, 13.000 rpm) and removal of flow-through, columns were centrifuged again (1 min, 13.000 rpm) to remove residual wash buffer. For elution 25 - 50 µl water was added, the columns were incubated for 1 min at RT and centrifuged (1 min, 13.000 rpm).

4.4.2 Isolation of plasmids in a preparative manner

4.4.2.1 “Midi” and “Maxi” preparation of plasmids (Qiagen)

Buffer P1 (resuspension buffer)	50 mM Tris/HCl, pH 8.0
	10 mM EDTA
	100 µg/ml RNase A

Buffer P2 (lysis buffer)	200 mM NaOH 1 % SDS
Buffer P3 (renaturation buffer)	3.0 M NH ₄ OAc
Buffer QBT (equilibration buffer)	750 mM NaCl 50 mM MOPS, pH 7.0 15 % Ethanol 0.15 % Triton X-100
Buffer QC (washing buffer)	1.0 M NaCl 50 mM MOPS pH 7.0 15 % Ethanol
Buffer QF (elution buffer)	1.25 M NaCl 50 mM Tris/HCl, pH 8.5 15 % Ethanol

Desired bacterial clones were inoculated in 50 ml for midi-preparation (or 250 ml LB-medium for maxi preparation) in a conical shaking flask (37°C, 180 rpm, overnight). The pellet was re-suspended in 4 ml (10 ml) buffer P1 and 4 ml (10 ml) P2 were added for lysis of bacteria (RT, 5 min). 4 ml (10 ml) pre-chilled buffer P3 was added and culture was incubated on ice for 20 min or in -20°C room for 5 min. After centrifugation (30 min, 13.000, 4°C), the supernatant containing the plasmidial DNA was poured on a wet paper filter into a Qiagen-tip 100 (500), which was treated before with 4 ml (10 ml) of buffer QBT. Tip was washed with 10 ml (30 ml) QC twice, which removes redundant RNA, proteins and low molecular weight components. Plasmid DNA was eluted with 5 ml (15 ml) buffer QF. DNA was precipitated with 3.5 ml (10.5 ml) of isopropanol solution and centrifuged (30 min, 13.000 rpm, 4°C). The forming white pellet was washed twice with 70 % ethanol and air-dried for 3-5 min. Depending on the pellet size, it was dissolved in 100 µl - 500 µl H₂O and concentration was determined at Nanodrop measuring machine.

4.4.3 DNA recovery from an agarose gel

4.4.3.1 QIAquick gel extraction kit

One volume of the excised and weighted piece of gel was provided with three volumes of buffer QG. The gel piece was melted in a shaking thermomixer for 10 min at 50°C. The liquid solution was applied to a column and centrifuged (1 min, 13.000 rpm, 4°C). 750 µl of washing buffer PE were added to the column and centrifuged (1 min, 13.000 rpm, 4°C). Centrifugation (1 min, 13.000 rpm, 4°C) was repeated to remove residual buffer PE. DNA was eluted from the membrane by pipetting 25 µl of H₂O direct on the top of the membrane and centrifuging (1 min, 13.000 rpm, 4°C).

4.4.4 Isolation of genomic DNA (gDNA) with QIAamp®

Cells grown on culture flasks or well plates were harvested into 1.5 ml vials. After that, they were centrifuged (5 min, 1.000 rpm, 4°C) to obtain a cell pellet. Supernatant was discarded and pellets were dissolved in 200 µl PBS. 20 µl of Qiagen Protease was pipetted into the cell suspension followed by addition of 200 µl AL buffer. The sample was pulse-vortexed for 15 sec. Both components are required for lysis of cells, which was done in a shaking Eppendorf thermomixer at 56°C for 10 min. 200 µl (100%) ethanol was added and the sample was pulse-vortexed again for 15 sec. Solution was added to a QIAamp Mini spin column (in a 2.0 ml collection tube) and centrifuged (1 min, 8.000 rpm). The filtrate was discarded and the column was provided with 500 µl buffer AW1 and centrifuged (1 min, 8.000 rpm). The filtrate was discarded and the column was provided with 500 µl of buffer AW2 and centrifuged (3 min, 14.000 rpm). To remove residual buffer, centrifugation step was repeated (1 min, 14.000 rpm). Column was placed into a 1.5 ml vial and 50 - 200 µl elution buffer AE was added. After 1 min of incubation, samples were centrifuged (1 min, 8.000 rpm) and DNA concentration and quality was measured.

4.5 Modification of DNA

4.5.1 Enzymatic restriction of DNA

Standard DNA manipulations were carried out according to Sambrook and colleagues (1989). For an analytical restriction reaction, an amount of 0.5 - 1 µg DNA dissolved in 10 µl H₂O was digested with 0.5 - 2 units restriction enzyme over 1 h at the optimal reaction temperature for the enzyme (usually 37°C). The reaction buffers (10x) were provided together with the restriction enzyme by the producing company (NEB or Fermentas). When required, BSA was added to the restriction reaction at a concentration of 100 µl/ml. The resulting DNA fragments were analyzed on a 1% agarose gel.

For a preparative DNA restriction 5 - 10 µg DNA were dissolved in 50 - 100 µl H₂O and DNA was digested with 5 - 10 µl (approximately 10% of end volume) enzyme. Cut DNA fragments were used for cloning, transfections or probe labeling.

4.5.2 Dephosphorylation of DNA-fragments

To avoid self-ligation of a DNA fragment digested with one restriction enzyme, the fragment was dephosphorylated at the 5' or 3'-end with alkaline phosphatase (Calf Intestine Phosphatase). The restricted and purified DNA fragment was eluted in 16 µl H₂O and mixed with 2 µl of alkaline phosphatase and 2 µl buffer. Dephosphorylation was done at 37°C for 60 min.

4.5.3 Fill in reaction of DNA-fragment with sticky ends

Restriction of DNA gives rise to sticky ends, which are not compatible to other sticky DNA fragments. In order to meet this Klenow fragment (obtained from Polymerase I) has a 3' → 5' exonuclease activity and a 5' → 3' polymerase activity and can fill up the 5'-protruding ends of DNA pieces with complementary dNTPs. 1 µg of DNA with sticky ends was treated with 1 U Klenow enzyme (NEB) in the presence of 3 µl dNTP-mix (25 mM for each nucleotide) and 5 µl Buffer II (NEB) in a total volume of 50 µl. The mixture was incubated for 15 min at 25°C and terminated by heat inactivation (20 min) at 75°C).

4.5.4 Ligation

Ligation, mediated by the T4-DNA-Ligase is essential for the connection of sticky or blunt DNA fragments for the generation of novel vectors. 10 - 14 µl of insert (restricted DNA, shorter fragment) was mixed with 2 - 4 µl of template (backbone of the vector, longer fragment) in a 1.5 ml vial. 2 µl of 10 x Ligase buffer and 2 µl of T4-DNA-Ligase were added to have a final volume of 20 µl. DNA-enzyme mix was incubated at 16°C for 1 - 2 h. Subsequently, 10 µl of the ligation mixture were transformed in 100 µl of chemo-competent bacteria (DH10B or Top10F') by heat-shock (ch. 4.3.6). Transformed bacteria were put in 300 µl LB-medium and were shaken for 20 min at 37°C in an Eppendorf thermomixer. Then they were spread onto an agar plate supplemented with corresponding antibiotic, which was incubated at 37°C overnight.

4.6 DNA analysis

4.6.1 Gel electrophoresis

The size of a linearized or excised DNA fragment or entire plasmid was determined by analysis on 1 % agarose gel. The required amount of agarose was dissolved in 1xTAE buffer, melted and poured into an electrophoresis chamber together with ethidium bromide solution (1g/ml). A volume of 10% v/v loading buffer was added to DNA samples at a ratio of 1/6. Samples were loaded onto the gel by using 1xTAE as running buffer. The electrophoresis was performed at 80-140 Volt/cm for about 30-60 min. The agarose gel was photographed under UV light with wavelength at 260 nm.

4.6.2 "Polymerase Chain Reaction" (PCR)

The Polymerase Chain Reaction (PCR) is a simple method to amplify a defined DNA fragment from plasmids, gDNA or cDNA. Specific short oligonucleotides (primers) are designed

to recognize and bind to margin 5' single strand sequences in the leading and lagging strand of single strand DNA. After that the primer gets elongated by DNA polymerase. This process is repeated for several times and can give rise to a 10^9 -fold amplification of the template DNA. Recently thermostable polymerases as Taq (*Thermophilus aquaticus*) were purified and applied to avoid denaturation of the enzyme during melting of the DNA strand, which occurs at high temperatures. Enzymes with a "proof reading" activity were developed to minimize error rate during elongation. The composition of a typical PCR reaction is indicated below.

2.5 µl	10 x PCR buffer
13 - 22.5 mM	MgCl ₂
500 nM	Tris-HCl, pH 9.2 (25°C)
160 mM	(NH ₄) ₂ SO ₄
100 - 200 µM	dNTP-Mix (contains all 4 nucleotides)
25 pmol	Primer A and B
H ₂ O	Millipore quality
1 - 3 U	Taq and Pwo polymerase

Most PCR reactions start with an initial denaturation step (5 min, 94°C) and are followed by three major steps, which are repeated for 12 – 50 times

1. Denaturation

During the denaturation (the DNA double strand melts yielding single strands) all enzymatic reactions are stopped (for example, the extension from the previous step). This step is performed usually at temperatures of 94°C - 95°C for 30 sec

2. Annealing

In these step primers, which are complementary to the 3'-end of the template DNA, anneal to the DNA at a lower temperature (approximately 55°C – 65°C) for 60 sec. After that the polymerase starts copying a DNA strand at the 3'-end of the primer sequence.

3. Elongation

The optimum temperature for elongation is 68°C - 72°C. The rate of synthesis is approximately 1.000 bp per minute and determines the time for copying a DNA strand (1 kb/ 1 min. After elongation is finished a new cycle starts with denaturation. If all cycles are completed reaction tube is cooled down to 16°C (RT) or 4°C.

General remarks: The concentration of MgCl₂ should be optimized for each primer pair. Wrong concentrations can give rise to unspecific products or a reduction of amplification. Primer should be 15 - 40 bp long and show no self-complementation at the 3'-end. The GC-content should be approximately at 50 %.

4.6.3 Specific mutagenesis of FRT sites with PCR

In order to create novel FRT-mutants for construction of exchange vectors with novel FRT mutants FRT sites were mutagenized with Expand Long Template PCR System (Roche). Primers were constructed to generate PCR products, which have a mutated spacer. These PCR products were cut by restriction and ligated into backbones.

4.6.4 Southern Blot analysis

Southern Blot is a molecular method to identify a gene sequence in a complex DNA mixture, for instance a whole genome of an organism.

4.6.4.1 Chemicals

Solution 1	0.5 M NaOH 1.0 M NaCl
Solution 2	0.5 M Tris-HCl pH 7.4 3.0 M NaCl
Solution 3	0.5 M NaOH
Solution 4	0.3 M Tris-HCl pH 7.5
Hybridizing solution	10 % Dextran sulfate 1 % SDS 1 M NaCl
Washing Solution 1	2x SSC
Washing Solution 2	2x SSC 0.1 % SDS
Washing Solution 3	1x SSC 0.1 % SDS
Washing Solution 4	0.1x SSC 0.1 % SDS
Stripping Solution	0.4 M NaOH 0.1x SSC 0.1 % SDS 0.2 M Tris pH 7.5

4.6.4.2 Restriction of gDNA and Blotting

10 µg of gDNA were digested with 4 µl of a restriction enzyme of choice and 4 µl of enzyme buffer (10x). Reaction was filled up with H₂O to 40 µl. Digestion was done overnight at 37°C and 3 µl of enzyme was added to the sample on day two. Samples were run on a 0.8% agarose gel in 1x TAE buffer at 24 - 30 V/cm overnight. At day three gel was treated with solution 1 and 2 for 20 min each. After that gel was treated with 10x SSC for 30 min. DNA was transferred to a positively charged Nylon membrane (Amersham) in 10x SSC. On a gel carrier plate a Whatman paper bridge soaked in 10x SSC was formed between two sides of buffer reservoir. On this the gel was placed upside down and the nylon membrane, soaked in water was put onto the gel. Air bubbles between the gel and membrane were removed carefully. On top of the membrane three Whatman papers were positioned. A 10 cm stack of dry tissue paper was added to absorb the buffer. The blot apparatus was covered with plastic foil, and a 0.5 kg weight was put on top.

The horizontal transfer of DNA to the membrane was done overnight. The nylon membrane was neutralized in solution 3 and 4 for 30 - 60 sec and baked at 80°C in an oven for 2 h before it was ready to use in the hybridization. (Pre-)hybridizations were done in hybridizing solution. Previously, the membrane had to be pre-hybridized for at least 90 min at 65°C in an appropriate incubator.

4.6.4.3 Radioactive labeling and hybridization

Labeling of DNA-probes was performed with DecaLabel™ DNA Labeling Kit (MBI Fermentas) 100 ng of the DNA-probe (length: 500 - 1000 bp) was provided with 10 µl decanucleotides in a 5x reaction buffer. Reaction was filled to 40 µl with H₂O. Mixture was vortexed and spun down in a centrifuge, followed by 10 min incubation at 100°C for denaturation of DNA. Probe was chilled on ice for 5 min. For radioactive labeling, 4 µl MixC, 5 µl of α[³²P]dCTP and for the filling reaction 1 µl of Klenow enzyme was added. The mixture was incubated in a thermomixer at 37°C for 15 min. the polymerase reaction was stopped by adding 5 µl 0.5 M EDTA pH 8.0 to the reaction. 45 µl of TE were added to gain final volume of 100 µl. Probe was purified with Mobi Spin Column S300. The column was vortexed and then centrifuged for 1 min at 2.700 rpm. The flow-through was discarded and the labeling mix was put on the column drop by drop and centrifuged through the column for 2 min at 2.700 rpm. 2 µl of the final probe were used to estimate activity by Cerenkov-counting at a scintillation counter.

For hybridization the labeled probe was denatured at 100°C for 5 min, then put on ice for 5 min and added to the pre-hybridized membrane. Hybridization was done overnight in a 65°C hot incubator.

After hybridization the membrane was washed to get rid of non-bound radioactive probe. The membrane was treated with washing solutions 1 - 4 for 10 min in a 60°C water bath. After each washing step, radioactive activity of the membrane was estimated. If the hand-counter determined an activity over 100 ips washing was continued till the activity dropped beyond 40 ips. After washing the blot was covered with a plastic bag foil and was welded. The blot was covered by a medical X-ray film (Fuji) and put in a x-ray exposition cassette (Rego). The blot was developed in an X-OMAT 1000 processor (Kodak).

For re-hybridization, the signals were eliminated by washing the blot with the stripping solution at 42° for 30 min. To check if the signal was completely removed, the stripped blot was

exposed again, until it was free of signal spots. Re-hybridization was performed as described above.

4.7 Eukaryotic cell culture

4.7.1 Cell lines

NIH/3T3 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Biochrom AG) supplemented with 10% inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 mM sodium pyruvate.

Clone O5 was cultured in the above described medium containing hygromycin B (150 U/ml) and Geneticin (G418) (500 U/ml) as selective agents. Cells were incubated at 37 °C with 5% CO₂ in a BBD 6220 incubator (Heraeus). Cells were passaged on a six well plate or on a 10 cm plate 3 days or 4 days after reaching 90% confluence.

4.7.2 Medium and buffer solutions for cell culture

All reagents were dissolved in "Millipore" water and sterile-filtrated (pore size of 2 µm).

Penicillin/Streptomycin solution (100x)	1.212 g Penicillin/200 ml (10000 U/ml), 2 g Streptomycin/200 ml (10 mg/ml), adjust to pH 7.0 with NaOH, stored at -20°C
Glutamine solution (100x)	29.23 g Glutamine dissolved to 1 l solution, stored at -20 °C
PBS	140 mM NaCl, 27 mM KCl, 7.2 mM Na ₂ HPO ₄ , 14.7 mM KH ₂ PO ₄ (pH 6.8 - 7.0), stored at 4°C
TEP	500 ml sterile PBS, 0.6 ml 0.5 M EDTA (final conc.: 6 mM), 15-20 ml Trypsin (0.1 - 0.2%; depending on the activity)
Puromycin	5 mg/ml in H ₂ O, stored at 4°C
Geneticin (G418)	100 mg/ml in H ₂ O, stored at -20°C
Hygromycin	650 U/ml in H ₂ O, stored at 4°C
Ganciclovir	10 mM/ml in H ₂ O, stored at 4°C

Gentamicin	5 mg/ml in H ₂ O, stored at -20°C
TE	10mM Tris/HCl, pH 8.0, 1 mM EDTA, pH 8.0
Medium for NIH3T3 cell line	Dulbecco's Modified Eagle's Medium (DMEM) 3.7 g/L NaHCO ₃ 4.5 g/L D-Glucose with stable glutamine with 10% fetal calf serum (FCS) Penicillin/Streptomycin solution (1x) Sodium pyruvate (1 mM)

4.7.3 Cell passage

Medium was changed every three days until the cells grew confluent for the next passage. When the cells had reached confluence on dishes, plates or flasks they were washed with 1x PBS once and were trypsinized with 1x TEP in the incubator (37°C) for several minutes. After that cells were suspended by pipetting and a portion of 1/20-1/10 was transferred to a new dish, flask or plate, which was previously provided with fresh medium.

4.7.4 Cell counting with Neubauer chamber

Cells were grown on plates or flasks until they reached confluence. Cells were washed with 2 ml (6 well plate) or 5 - 10 ml (25 cm² and 75 cm² flask) 1x PBS once and were trypsinized with 0.5 ml (6 well plate), 1 ml (25 cm² flask) or 2 ml (75 cm² flask) 1x TEP. Cells were suspended gently in 5 - 25 ml DMEM Medium and transferred to 50 ml Nunc tubes. 20 µl of the cell suspension was pipetted between the Neubauer counting chamber and a previously attached cover-slip. Viable cells of 4 squares were counted at four different positions. The average cell number was multiplied with 1E4 to receive the cell number/ml.

4.7.5 Cryopreservation and thawing of eukaryotic cell lines

Cells were grown on 6 well plates until they reached confluence (about 5E6 cells). Cells were washed with 2 ml 1x PBS once and trypsinized with 500 µl 1x TEP. Cells were suspended in 9 ml medium and then centrifuged (5 min, 1.000 rpm). Subsequently, supernatant was removed and pellet was resuspended in 1 ml FCS containing 10 % DMSO. Cell suspension was pipetted into cryo-vials (Bio-Freeze-Vials, Nunc) and put on ice immediately. Cryo-vials were transferred into freezing container (Nalgene™, Cryo 1°C) and deposited into a -70°C fridge. After 1 day cryo-vials were transferred into liquid nitrogen container for long-term storage. The frozen vials were thawed in a 37°C water bath for 1 minute. 9 ml pre-warmed

medium was mixed with the thawed cells in a 15 ml reaction tube. In order to remove residual DMSO cells were centrifuged for 5 min at 1.000 rpm. The supernatant was discarded and the cell pellet was re-suspended in 5 ml medium and pipetted into a little flask.

4.8 Gene transfer

4.8.1 Electroporation

Logarithmically growing, semiconfluent cells (1E6) were collected by trypsinization and re-suspended in 0.7 ml of pre-chilled DMEM together with 1 µg of linearized DNA. The cell-DNA mixture was pulsed at 320 V and 985 µF with infinite resistance from a Bio-Rad Gene Pulser and Pulse Controller (Bio-Rad, Hercules, CA). Electroporated cells were plated on a 10 cm plate containing 10 ml of DMEM. Twentyfour hours after electroporation, the culture medium was changed to a medium with selection drugs.

4.8.1.1 Construction of multiplexing-RMCE competent cell lines

The NIH/3T3 cell line N1 (Goetze *et al.*, 2005), which bears one copy of F_3 -hyg^{tk}- F_{wt} , was used for electroporation. One microgramme of the $ScaI$ -linearized vector DNA F_{13} -tkneo- $F_{14}+220$ was suspended with 1E6 trypsinized cells and electroporated under the conditions described above. After 14 days of double selection with hygromycin B (150 U/ml) and Geneticin (500 U/ml) on 10 cm plates, single cells from cell pools were seeded into 96 well plates by limiting dilution. Growing cell clones were analyzed for the copy number of F_{13} -tkneo- $F_{14}+220$ in Southern Blot studies. Cell clone O5, which harbors one copy of F_{13} -tkneo- F_{14} in addition to F_3 -hyg^{tk}- F_{wt} , was further investigated in multiplexing-RMCE experiments (ch. 4.10.4).

4.8.2 Transfections for transient RMCE

One day prior to transfection, NIH/3T3 cells (1.5E5 per well) were seeded on a 6 well plate. Four hours before transfection, the culture medium was exchanged. The cells were co-transfected with 2.4 µg of $F'-F''$ P vector, 0.8 µg of $F-F''$ ΔP vector, and 1 µg of Flpo-puro (1 µg of pBSpac-Δp as negative control) in accordance with the Metafectene transfection protocol. One day post transfection, the cells were washed with PBS, and the culture medium was changed to DMEM with the selective agent puromycin (2.5 µg/ml) for 24 h. Two days post transfection the cells were harvested and FACS-analyzed.

4.8.3 Transfections for multiplexing-RMCE

One day prior to transfection, NIH/3T3-derived clone O5 cells (2.0E5 per well) were seeded on 6 well plates. Four hours before transfection, the culture medium was exchanged with a medium without selective antibiotics. The cells were cotransfected with 1.5 µg of both exchange vectors and 1 µg of Flpo-puro (1 µg of BSpac-Δp as negative control) in accordance with the Metafectene transfection protocol. One day post transfection, the cells were washed with PBS, and the culture medium was changed to DMEM with the selective agent puromycin (2.5 µg/ml) for 24 h. Two days after transfection, the cells were harvested and sorted at MoFlo XDP for eGFP/DsRed coexpressers (ch. 4.9.2) twice within 7 days. Double-sorted cells (2E4) were seeded on 10 cm plates. After 2 days, the medium was changed to DMEM with 100 nM Ganc, a negative selection drug. After 14 days of selection, growing cell clones were pooled by trypsinization and further analyzed for RMCE with PCR and Southern blot analysis.

4.9 Fluorescence-activated Cell Sorting (FACS)

4.9.1 Scanning by FACSCalibur

EPICS Buffer

2% inactivated FCS (FCS inactivated in a 56°C water bath for 30 min) in PBS

The expression of eGFP and DsRed was analyzed by a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson, San Jose, CA). Confluent cells on a 6 well plate were washed with 2 ml PBS and collected by trypsinization with 500 µl of 1x TEP. The suspension was mixed with 500 µl PBS and centrifuged (5 min, 1.000 rpm, 4°C). The supernatant was removed. The pellet was re-suspended in 400 µl EPICS containing 2% deactivated fetal calf serum. The excitation wavelength for eGFP is 489 nm, and emission was detected at 508 nm (on FI-1). The DsRed excitation wavelength is 558 nm, and emission was detected at 583 nm (on FI-2). The eGFP/DsRed coexpression was evaluated in a FI-2 versus FI-1 dot plot on live cells. 1E5 events were collected. Data were acquired and analyzed by CellQuest™ Pro.

4.9.2 Sorting by MoFlo XDP

The confluent cells on a 6 well plate were collected as described above. Single cells or cell populations expressing eGFP/DsRed were separated by a fluorescence-activated cell sorter (MoFlo XDP; Beckman Coulter). The suspended cells were passed through a 100 µm gauze to exclude aggregated cells. eGFP expressing cells were detected and sorted at 488 nm on F11-A, whereas DsRed expressing cells were detected and sorted at a wavelength of 561 nm. Sorting of eGFP/DsRed-coexpressing cells was applicable without compensation steps because the MoFlo XDP sorter was equipped with a blue laser for sorting eGFP and with a yellow laser for sorting DsRed expressing cells. Doublets were excluded via an FSC-H versus FSC-A dot plot. The sorting gate was a combination of the live cell gate, the doublet discrimination gate, and the dot plot gate on FSC-A versus F11-A. The sorted cells were in-

cubated for several days in the medium with 5 µg/ml Gentamicin. Data were acquired and analyzed by FlowJo 7.2.2 software (Tree Star, Inc.).

4.10 Plasmids and Oligonucleotides

4.10.1 Plasmids

4.10.1.1 Existing plasmids used in this study

Flpe-puro: expression plasmid for Flpe (flippase “enhanced”) under the control of the CAGGS promoter (Buchholz *et al.*, 1998)

pMBC-1: this vector has been described previously (Dirks *et al.*, 1994)

pBHEluc: expression plasmid for firefly luciferase under the control of the SV40 enhancer/promoter.

pF₃-hyg^{tk}-F_{wt}: The hygromycin thymidine kinase fusion gene (*hyg^{tk}*) under control of the HSV-thymidine kinase promoter is flanked by F₃ and F_{wt} sites (Schlake & Bode, 1994).

F₃-cmv-d2eGFP-F_{wt}: the construct has been described previously (Qiao *et al.*, 2009).

pDsRed-Express-N1: This vector was purchased by Clontech.

pPGKFLPObpA: This vector expresses *de novo* codon-optimized Flp (Flpo), and was constructed by Raymond *et al.*, (2008) and purchased by Addgene.

pRRL.PPT.SF.GFP.pre: This construct is a SIN third generation human immunodeficiency virus-1 lentiviral vector (Zufferey *et al.*, 1998).

pBSpac-Δp: This is a puromycin-expressing vector used in place of the Flpo-puro expression vector as negative control for transient RMCE and multiplexing-RMCE experiments.

pHyg^{tk}fus: This vector has been described previously (Karreman *et al.*, 1996)

pNeoβGal: This vector has been described previously (O’ Gorman *et al.*, 1991)

pSBC2tkneoL This vector has been described previously (Wodarczyk, C., Dissertation, TU-Braunschweig, 2003)

4.10.1.2 Plasmids constructed in this study

Flpo-puro

Flpo was PCR-amplified from the template plasmid pPGKFLPObpA (Addgene) with primers (P3132/P3133). Flpe was excised from Flpe-puro with XbaI and PaeI. PCR product was cut with XbaI and PaeI and ligated with the backbone sequence to yield Flpo-puro.

F₁₁-tkneo-F₁₂+220 and F₁₃-tkneo-F₁₄+220.

The *tkneo* fusion gene was PCR-amplified from pSBC2tkneoL with primers (5TKneo/3TKneo). PCR product and F₃-hygk-F_{wt} were cut with BamHI and ClaI and ligated to yield the intermediate cloning product F₃-tkneo-F_{wt}. Mutant FRT F₁₁ site was amplified by PCR from pNeoβGal with primers (FRT11NHETK5/FRT11TK3) and cut by NheI. The preliminary vector was opened in 3' position to the SV40 enhancer origin promoter by NheI, and the FRT F₁₁ fragment was inserted. Mutant FRT F₁₂ site was amplified by PCR from pNeoβGal with primers (FRT11TK5/FRT12TK3), cut by BglII/SalI and then ligated into the opened (BglII/SalI) backbone. In order to provide a unique priming sequence for the final PCR analysis, a 224-bp fragment from pHygkfus with BamHI/NdeI was excised (marked by the term "+220"). F₁₁-tkneo-F₁₂ was opened with BamHI/NdeI and the fragment was inserted in 3'-position relative to the F₁₂ site to yield F₁₁-tkneo-F₁₂+220. This construct served as a template for the construction of F₁₃-tkneo-F₁₄+220.

FRT F₁₃ site was amplified by PCR with primers (P3924/P3925) from pNeoβGal and cut by NheI. A fragment containing the F₁₁ site was excised from F₁₁-tkneo-F₁₂+220 by NheI, and F₁₃ site fragment was inserted in this locus. Mutant FRT F₁₄ site was amplified by PCR with primers (P3927/P3930) from pNeoβGal, cut by BglII/SalI and then ligated into the opened (BglII/SalI) backbone.

F₁₁-RFPex-F₁₂ and F₁₃-RFPex-F₁₄:

rfpex gene was amplified from the template vector pDsRed-Express-N1 with primers (RFPex5NXHO/RFPex3N) and cut with BglII/XhoI. In order to exchange *tkneo* for *rfpex*, F₁₁-tkneo-F₁₂ was cut with BglII/XhoI and ligated with cut PCR fragment.

F'-F'' P and F'-F''ΔP.

F'-F'' P: FRT sites were PCR-mutagenized and amplified from the template vector pNeoβGal. FRT sites to be inserted in 5'-position (F' position) were amplified with following primers:

Tab. 4.1 Primer pairs needed for amplification of wildtype or mutant FRTs to be inserted in 5'

Primer pair	Amplification of wild type or mutant FRTs
ST_FRTWTNheI5/ST_FRTWTNcoI3	F _{wt}
ST_FRTWTNheI5/ST_FRTF3NcoI3	F ₃
ST_FRTWTNheI5/ST_FRTF5NcoI3	F ₅
ST_FRTWTNheI5/ST_FRTF13NcoI3	F ₁₃
ST_FRTWTNheI5/ST_FRTF14NcoI3	F ₁₄

Backbones and FRTs to be inserted in position F' were cut with NheI/NcoI and ligated. FRT sites to be inserted in 3'-(F'' position) were amplified with following primers:

Tab. 4.2 Primer pairs needed for amplification of wild type or mutant FRTs to be inserted in 3'

Primer pair	Amplification of wild type or mutant FRTs
ST_FRTWTBglII5/ST_FRTWTSalI3	F _{wt}
ST_FRTWTBglII5/ST_FRTF14SalI3	F ₁₄
ST_FRTWTBglII5/ST_FRTF15SalI3	F ₁₅

Backbones and FRTs to be inserted in position F'' were cut with BglII/SalI and ligated.

F₁₃-F₁₄ P: SV40-enhancer/promoter-containing fragment was excised from F₁₃-RFPex-F₁₄ with XhoI/SalI and ligated to obtain the intermediate vector F₁₃-ΔSV40-RFPex-F₁₄. SV40 enhancer/promoter was amplified by PCR from the template DNA F₁₁-tkneo-F₁₂ with primers (ST_SV40NheI5/ST_SV40NheI3).and cut with NheI. The intermediate vector was opened with NheI and ligated with the SV40 promoter fragment.

F'-F'' ΔP: SV40: enhancer/promoter was excised from F'-F'' P-type vectors with NheI, and the PCR-amplified primers (ST_polyANheI5/ST_polyANheI3) late SV40 polyA sequence was inserted in the NheI cut backbone in an inverse orientation. *rfpex* gene replacement by *egfp* was performed similarly to F13-F14 ΔP.

F₁₃-F₁₄ ΔP: To diminish cryptic background expression activities on the promoter free donor originating from a promoter resembling sequences upstream of the silent transgene, we opened F₁₃-ΔSV40RFPex-F₁₄ 5' to the F₁₃ site with NheI and ligated it with PCR-amplified primers (ST_polyANheI5/ST_polyANheI3) and NheI-cut SV40 late polyA fragment from the template DNA F₁₃-RFPex-F₁₄ to yield the intermediate vector pAi-F₁₃RFPexF₁₄. The background activity of this vector could be reduced considerably by inserting the given poly A sequence in inverse orientation. In order to change the *rfpex* open reading frame (ORF) for *egfp*, NotI/NcoI cut the intermediate vector and the cloning vector pRRL.PPT.SF.GFP.pre. The eGFP ORF was ligated into the backbone.

4.11 Oligonucleotides

Oligonucleotides were synthesized by the company Eurofins MWG Operon

Oligonucleotides used for cloning vectors:

P3132	5'-CGC AGC TTC TAG AAT GGC TCC TAA GAA GAA G-3'
P3133	5'-CCG TCT TAA TTA ATC AGA TCC GCC TGT TGA TGT-3'
5TKneo	5'-CGC AGC ATC GAT AAG CTA GCT AGC TTC TGT GG-3'
3TKneo	5'-GCT AGG ATC CGT CGA CCT AGA GAT CTT ACC ACA TTT GTA GAG G-3'

FRT11NHETK5	5'-CGC AGC TGC TAG CCC TTC GCC AGG GGC ATC TT-3'
FRT11TK5	5'-CGC AGC TAG ATC TAT CGA TCC TTC GCC AGG GGC-3'
FRT11TK3	5'-CGT CGC TAG CGT CGA CGA AGT TCC TAT ACT TAG TTC AGA ATA G-3'
FRT12TK3	5'-CGT CGC TAG CGT CGA CGA AGT TCC TAT ACT TCA GAA AGA ATA G-3'
P3924	5'-CGC AGC GCT AGC CCT TCG CCA GGG GCA TCT T-3'
P3925	5'-CCG TCG GCT AGC GTC GAC GAA GTT CCT ATA CTT ATA TGA GAA TAG-3'
P3927	5'-CGC AGC AGA TCT ATC GAT CCT TCG CCA GGG-3'
P3930	5'-CCG TCG CAT ATG GAA GTT CCT ATA CTT CTG ATA GAA TAG-3'
RFPex5NXHO	5'-CGC AGC CTC GAG CGG GAT CCA CCG GTC GCC-3'
RFPex3N	5'-GGC TAG AGA TCT CCA CAA CTA GAA TGC AGT G-3'
ST_SV40NheI5	5'-CGC AGC GCT AGC CTG TGG AAT GTG GTC AG-3'
ST_SV40NheI3	5'-CCG TCG GCT AGC TTT TTG CAA AAG CCT-3'
ST_FRTWTNheI5	5'-CGC AGC GCT AGC CCT TCG CCA GGG GCT ACC-3'
ST_FRTWTBglI5	5'-CGC AGC AGA TCT CCT TCG CCA GGG GCT ACC-3'
ST_FRTWTNcoI3	5'-CCG TCG CCA TGG GAA GTT CCT ATA CTT TCT AGA GAA TAG-3'
ST_FRTWTSaI3	5'- CCG TCG GTC GAC GAA GTT CCT ATA CTT TCT AGA GAA TAG-3'
ST_FRTF3NcoI3	5'-CCG TCG CCA TGG GAA GTT CCT ATA CTA TTT GAA GAA TAG-3'
ST_FRTF13NcoI3	5'-CCG TCG CCA TGG GAA GTT CCT ATA CTT ATA TGA GAA TAG-3'
ST_FRTF14NcoI3	5'-CCG TCG CCA TGG GAA GTT CCT ATA CTT CTG ATA GAA TAG-3'
ST_FRTF14SaI3	5'-CCG TCG GTC GAC GAA GTT CCT ATA CTT CTG ATA GAA TAG-3'
ST_FRTF15SaI3	5'- CCG TCG GTC GAC GAA GTT CCT ATA CTC CTA TAA GAA TAG -3'
ST_polyANheI5	5'-CGC AGC GCT AGC CAG ACA TGA TAA GAT ACA-3'
ST_polyANheI3	5'-CCG TCG GCT AGC TAC CAC ATT TGT AGA GGT-3'

Primers used for multiplexing-RMCE

RMCECHECK5	5'-GCG GCC GCG ACT CTA GAT CAT AAT CAG-3'
RMCEUP3	5'-TAA CGC CAG GGT TTT CCC AGT CAC GAC-3'
f3f.rmce.nov.2.5	5'-TTT ATG CTT CCG GCT CGT ATG TTG TGT-3'
f3f.rmce.nov.3	5'-GGA AAT CCC CGT GAG TCA AAC CGC TAT-3'

5 Abbreviations

aa	amino acid
A	adenosine
α	Alpha
ac	acetate
<i>amp</i>	<i>ampicillin resistance gene</i>
AP	alkaline phosphatase
ATCC	american type culture collection
ATP	adenosine triphosphate
Arg	arginine
attP	attachment site of phage
attB	attachment site of bacteria
att	attachment site for Φ C31
β	beta
β -Gal	β -Galactosidase
<i>β-geo</i>	fusion gene, composed of <i>lacZ</i> gene and <i>neo</i> gene
BHK	baby hamster kidney cells
bp	base pairs
BSA	bovine serum albumin
C	cytosine
$^{\circ}\text{C}$	degree in Celsius
CAGGS	promoter constituted by the cytomegalovirus-enhancer, β -actin promoter and the β -globin poly(A)-signal
cDNA	complementary DNA
ch	chapter
CHO	Chinese hamster ovary cells
cf	confer
CIP	calf intestine phosphatase
cmv	cytomegalovirus promoter
CpG	cytosine-guanine-dinucleotide
Cre	causes recombination (Cre-recombinase)
Δ	delta, deletion
d2eGFP	destabilized enhanced green fluorescent protein
<i>d2egfp</i>	<i>destabilized enhanced green fluorescent protein gene</i>
dNTP	deoxyribonucleotidetriphosphate (A, C, G, T)
DME	Dulbecco's modified eagle medium

DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
<i>E.coli</i>	<i>Escherichia coli</i>
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
<i>egfp</i>	<i>enhanced green fluorescent protein gene</i>
<i>elk1</i>	<i>ets like gene 1</i>
ER	estrogen receptor
ES	embryonic stem
F_{wt}	wild type FRT site
F_n	mutant FRT site
FACS	fluorescence-activated cell sorter or sorting
FCS	fetal calf serum
<i>flp</i>	<i>flp recombinase gene</i>
Flp	Flp recombinase (Flippase)
Flpe	enhanced Flp recombinase
Flpo	mouse codon optimized enhanced Flp recombinase
<i>fluc</i>	<i>firefly-luciferase gene</i>
fmol	femtomol
FRT	Flp recognition target
g	gramme
G	guanine
G418	aminoglycoside-2'-deoxystreptine (Gentamicin derivate)
Ganc	Ganciclovir
Ganc ^r	Ganciclovir resistant
gDNA	genomic DNA
GFP	green fluorescent protein
GOI	gene of interest
Y	gamma
h	hour
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
His	histidine
hyg ^r	hygromycin resistant
<i>hygk</i>	hygromycin and thymidine kinase fusion gene

HygTk	hygromycin and thymidine kinase fusion protein
HR	homologous recombination
Int	Integrase
iPS	induced pluripotent stem
iPS	induced pluripotent stem
IRES	internal ribosome entry site
kb	1000 base
kbp	kilobase pair
kDa	kilodalton
kV	kilovolt
l	liter
λ	lambda
<i>lacZ</i>	<i>β-galactosidase</i> gene
LB	media for cultivation of <i>E.coli</i>
LTR	long terminal repeat
loxP	locus of crossing over of P1 (target sequence of the Cre recombinase)
lox	loxP site
<i>lrp</i>	<i>low density lipoprotein receptor-related protein 1</i> gene
μ	My
μ F	microfarad
μ g	microgram
μ l	micro liter
μ m	micrometer
μ M	micro molar
M	molar
mA	milli ampère
mar	matrix attachment region
MEF	murine embryonic fibroblast
mg	milli gramme
min	minute
ml	Milliliter
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger RNA
ms	millisecond

<i>neo</i>	neomycin-phosphotransferase-gene
<i>neo^r</i>	neomycin resistant
<i>neotk</i>	fusion gene of neomycin-phosphotransferase-gene and thymidine-kinase-gene
NeoTk	fusion protein of neomycin-phosphotransferase and thymidine-kinase
nm	nanometer
nt	nucleotide
ORF	open reading frame
ori	origin of replication
p	plasmid
Φ	Phi
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGK	promoter of phosphoglycerate kinase
pH	the negative logarithm (base 10) of the molar concentration of dissolved hydronium ions
pmol	10 ⁻¹² mol
<i>rfp</i>	red fluorescent protein-gene
<i>rfpex</i>	red fluorescent protein express-gene
RFP	red fluorescent protein
RMCE	recombinase-mediated cassette exchange
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
rs	recognition site
RT	room temperature
s	second
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SA	splice acceptor
SAR	scaffold attachment region
SDS	sodium lauryl sulfate
SEAP	secreted alkaline phosphatase
sec	second

SIN	self-inactivating
S/MAR	scaffold/matrix attachment region
SSC	mixture of 10x sodium chloride and 1x trisodium citrate
SSR	site specific recombination
SV40	Simian Virus 40
T	thymidine
tab	table
TAE	tris-acetate-EDTA buffer
TBE	tris-boric acid-EDTA buffer
TB	media for cultivation of <i>E. coli</i>
TE	tris/EDTA buffer
TEP	trypsin/EDTA/PBS buffer
tk	HSV-thymidine-kinase gene
Tk	HSV-thymidine-kinase protein
tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
U	units
U	uridine
UV	ultraviolet
V	Volt
vol	volume
v/v	volume/volume (percentage of volume)
w/v	weight/volume (percentage of weight)
wt	wild type

6 Literatures

Abremski, K. and R. Hoess (1984). "Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein." *J Biol Chem* **259**(3): 1509-14.

Amin, A., H. Roca, et al. (1991). "Synapsis, strand scission, and strand exchange induced by the FLP recombinase: analysis with half-FRT sites." *Mol Cell Biol* **11**(9): 4497-508.

Andrews, B. J., G. A. Proteau, et al. (1985). "The FLP recombinase of the 2 micron circle DNA of yeast: interaction with its target sequences." *Cell* **40**(4): 795-803.

Aranda, M., C. Kanellopoulou, et al. (2001). "Altered directionality in the Cre-LoxP site-specific recombination pathway." *J Mol Biol* **311**(3): 453-9.

Argos, P., A. Landy, et al. (1986). "The integrase family of site-specific recombinases: regional similarities and global diversity." *Embo J* **5**(2): 433-40.

Artelt, P., R. Grannemann, et al. (1991). "The prokaryotic neomycin-resistance-encoding gene acts as a transcriptional silencer in eukaryotic cells." *Gene* **99**(2): 249-54.

Askew, G. R., T. Doetschman, et al. (1993). "Site-directed point mutations in embryonic stem cells: a gene-targeting tag-and-exchange strategy." *Mol Cell Biol* **13**(7): 4115-24.

Austin, S., M. Ziese, et al. (1981). "A novel role for site-specific recombination in maintenance of bacterial replicons." *Cell* **25**(3): 729-36.

Baer, A. and J. Bode (2001). "Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes." *Curr Opin Biotechnol* **12**(5): 473-80.

Bateman, J. R., A. M. Lee, et al. (2006). "Site-specific transformation of *Drosophila* via phiC31 integrase-mediated cassette exchange." *Genetics* **173**(2): 769-77.

Bethke, B. and B. Sauer (1997). "Segmental genomic replacement by Cre-mediated recombination: genotoxic stress activation of the p53 promoter in single-copy transformants." *Nucleic Acids Res* **25**(14): 2828-34.

Bi, W. L., Stambrook, P.J. et al., (1993). "In Vitro Evidence That Metabolic Cooperation Is Responsible for the Bystander Effect Observed with HSV *tk* Retroviral Gene Therapy" *Human Gene Therapy*, **4**(6): 725-731.

Bode, J., C. Benham, et al. (2000). "Transcriptional augmentation: modulation of gene expression by scaffold/matrix-attached regions (S/MAR elements)." *Crit Rev Eukaryot Gene Expr* **10**(1): 73-90.

Bode, J., T. Schlake, et al. (2000). "The transgeneticist's toolbox: novel methods for the targeted modification of eukaryotic genomes." *Biol Chem* **381**(9-10): 801-13.

- Bolusani, S., C. H. Ma, et al.** (2006). "Evolution of variants of yeast site-specific recombinase Flp that utilize native genomic sequences as recombination target sites." *Nucleic Acids Res* **34(18)**: 5259-69.
- Bouhassira, E. E., K. Westerman, et al.** (1997). "Transcriptional behavior of LCR enhancer elements integrated at the same chromosomal locus by recombinase-mediated cassette exchange." *Blood* **90(9)**: 3332-44.
- Bradley, A., M. Evans, et al.** (1984). "Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines." *Nature* **309(5965)**: 255-6.
- Bruckner, R. C. and M. M. Cox** (1986). "Specific contacts between the FLP protein of the yeast 2-micron plasmid and its recombination site." *J Biol Chem* **261(25)**: 11798-807.
- Buchholz, F., P. O. Angrand, et al.** (1998). "Improved properties of FLP recombinase evolved by cycling mutagenesis." *Nat Biotechnol* **16(7)**: 657-62.
- Capeocchi, M. R.** (1989). "Altering the genome by homologous recombination." *Science* **244(4910)**: 1288-92.
- Cesari, F., V. Rennekampff, et al.** (2004). "Elk-1 knock-out mice engineered by Flp recombinase-mediated cassette exchange." *Genesis* **38(2)**: 87-92.
- Chen, Y. and P. A. Rice** (2003). "New insight into site-specific recombination from Flp recombinase-DNA structures." *Annu Rev Biophys Biomol Struct* **32**: 135-59.
- Cobellis, G., G. Nicolaus, et al.** (2005). "Tagging genes with cassette-exchange sites." *Nucleic Acids Res* **33(4)**: e44.
- Cox, M. M.** (1983). "The FLP protein of the yeast 2-microns plasmid: expression of a eukaryotic genetic recombination system in Escherichia coli." *Proc Natl Acad Sci U S A* **80(14)**: 4223-7.
- Dirks, W., F. Schaper, et al.** (1994). "A multifunctional vector family for gene expression in mammalian cells." *Gene* **149(2)**: 387-8.
- Doetschman, T., R. G. Gregg, et al.** (1987). "Targetted correction of a mutant HPRT gene in mouse embryonic stem cells." *Nature* **330(6148)**: 576-8.
- Dymecki, S. M.** (1996). "Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice." *Proc Natl Acad Sci U S A* **93(12)**: 6191-6.
- Evans, M. J. and M. H. Kaufman** (1981). "Establishment in culture of pluripotential cells from mouse embryos." *Nature* **292(5819)**: 154-6.
- Feng, Y. Q., J. Seibler, et al.** (1999). "Site-specific chromosomal integration in mammalian cells: highly efficient CRE recombinase-mediated cassette exchange." *J Mol Biol* **292(4)**: 779-85.

- Fiering, S., E. Epner, et al.** (1995). "Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta-globin locus." *Genes Dev* **9**(18): 2203-13.
- Friedrich, G. and P. Soriano** (1991). "Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice." *Genes Dev* **5**(9): 1513-23.
- Futcher, A. B.** (1986). "Copy number amplification of the 2 micron circle plasmid of *Saccharomyces cerevisiae*." *J Theor Biol* **119**(2): 197-204.
- Goetze, S., A. Baer, et al.** (2005). "Performance of genomic bordering elements at predefined genomic loci." *Mol Cell Biol* **25**(6): 2260-72.
- Golic, K. G. and S. Lindquist** (1989). "The FLP recombinase of yeast catalyzes site-specific recombination in the *Drosophila* genome." *Cell* **59**(3): 499-509.
- Gu, H., J. D. Marth, et al.** (1994). "Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting." *Science* **265**(5168): 103-6.
- Guo, F., D. N. Gopaul, et al.** (1997). "Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse." *Nature* **389**(6646): 40-6.
- Haber, J. E., G. Ira, et al.** (2004). "Repairing a double-strand chromosome break by homologous recombination: revisiting Robin Holliday's model." *Philos Trans R Soc Lond B Biol Sci* **359**(1441): 79-86.
- Hartley, J. L. and J. E. Donelson** (1980). "Nucleotide sequence of the yeast plasmid." *Nature* **286**(5776): 860-5.
- Higashi, A. Y., T. Ikawa, et al.** (2009). "Direct hematological toxicity and illegitimate chromosomal recombination caused by the systemic activation of CreERT2." *J Immunol* **182**(9): 5633-40.
- Hoess, R. H. and K. Abremski** (1985). "Mechanism of strand cleavage and exchange in the Cre-lox site-specific recombination system." *J Mol Biol* **181**(3): 351-62.
- Hoess, R. H., A. Wierzbicki, et al.** (1986). "The role of the loxP spacer region in P1 site-specific recombination." *Nucleic Acids Res* **14**(5): 2287-300.
- Hoess, R. H., M. Ziese, et al.** (1982). "P1 site-specific recombination: nucleotide sequence of the recombining sites." *Proc Natl Acad Sci U S A* **79**(11): 3398-402.
- Huang, L. C., E. A. Wood, et al.** (1991). "A bacterial model system for chromosomal targeting." *Nucleic Acids Res* **19**(3): 443-8.
- Ivics, Z., M. A. Li, et al.** (2009). "Transposon-mediated genome manipulation in vertebrates." *Nat Methods* **6**(6): 415-22.
- Jae Huh, W., I. U. Mysorekar, et al.** "Inducible activation of Cre recombinase in adult mice causes gastric epithelial atrophy, metaplasia, and regenerative changes in the absence of "floxed" alleles." *Am J Physiol Gastrointest Liver Physiol* **299**(2): G368-80.

- Jayaram, M.** (1985). "Two-micrometer circle site-specific recombination: the minimal substrate and the possible role of flanking sequences." *Proc Natl Acad Sci U S A* **82**(17): 5875-9.
- Jung, S., K. Rajewsky, et al.** (1993). "Shutdown of class switch recombination by deletion of a switch region control element." *Science* **259**(5097): 984-7.
- Jung, U. J., S. Park, et al.** (2007). "Analysis of spacer regions derived from intramolecular recombination between heterologous loxP sites." *Biochem Biophys Res Commun* **363**(1): 183-9.
- Karreman, S., H. Hauser, et al.** (1996). "On the use of double FLP recognition targets (FRTs) in the LTR of retroviruses for the construction of high producer cell lines." *Nucleic Acids Res* **24**(9): 1616-24.
- Kay, M. A., J. C. Glorioso, et al.** (2001). "Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics." *Nat Med* **7**(1): 33-40.
- Kilby, N. J., M. R. Snaith, et al.** (1993). "Site-specific recombinases: tools for genome engineering." *Trends Genet* **9**(12): 413-21.
- Kim, C. G., E. M. Epner, et al.** (1992). "Inactivation of the human beta-globin gene by targeted insertion into the beta-globin locus control region." *Genes Dev* **6**(6): 928-38.
- Kistner, A., M. Gossen, et al.** (1996). "Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice." *Proc Natl Acad Sci U S A* **93**(20): 10933-8.
- Klehr, D., K. Maass, et al.** (1991). "Scaffold-attached regions from the human interferon beta domain can be used to enhance the stable expression of genes under the control of various promoters." *Biochemistry* **30**(5): 1264-70.
- Kolb, A. F.** (2001). "Selection-marker-free modification of the murine beta-casein gene using a lox2272 [correction of lox2722] site." *Anal Biochem* **290**(2): 260-71.
- Kolb, A. F.** (2002). "Structure and regulation of the murine gamma-casein gene." *Biochim Biophys Acta* **1579**(2-3): 101-16.
- Konsolaki, M., M. Sanicola, et al.** (1992). "FLP-mediated intermolecular recombination in the cytoplasm of Drosophila embryos." *New Biol* **4**(5): 551-7.
- Kranz, A., J. Fu, et al.** "An improved Flp deleter mouse in C57Bl/6 based on Flpo recombinase." *Genesis* **48**(8): 512-20.
- Kuhn, R. and F. Schwenk** (1997). "Advances in gene targeting methods." *Curr Opin Immunol* **9**(2): 183-8.
- Kyba, M., R. C. Perlingeiro, et al.** (2002). "HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors." *Cell* **109**(1): 29-37.

- Lakso, M., B. Sauer, et al.** (1992). "Targeted oncogene activation by site-specific recombination in transgenic mice." *Proc Natl Acad Sci U S A* **89(14)**: 6232-6.
- Langer, S. J., A. P. Ghafoori, et al.** (2002). "A genetic screen identifies novel non-compatible loxP sites." *Nucleic Acids Res* **30(14)**: 3067-77.
- Lauth, M., K. Moerl, et al.** (2000). "Characterization of Cre-mediated cassette exchange after plasmid microinjection in fertilized mouse oocytes." *Genesis* **27(4)**: 153-8.
- Lauth, M., F. Spreafico, et al.** (2002). "Stable and efficient cassette exchange under non-selectable conditions by combined use of two site-specific recombinases." *Nucleic Acids Res* **30(21)**: e115.
- Lee J., Whang I., Lee J. and Jayaram M.** (1994) Directed protein replacement in recombination full sites reveals trans-horizontal DNA cleavage by Flp recombinase. *EMBO J* **13(22)**: 5346–5354
- Lee J., Jayaram M. and Grainge I.** (1999) Wild-type Flp recombinase cleaves DNA in trans The *EMBO J* **18**, 784 - 791
- Lee, G. and I. Saito** (1998). "Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination." *Gene* **216(1)**: 55-65.
- Li, X., X. Zhao, et al.** (1998). "Generation of destabilized green fluorescent protein as a transcription reporter." *J Biol Chem* **273(52)**: 34970-5.
- Lin, F. L., K. Sperle, et al.** (1985). "Recombination in mouse L cells between DNA introduced into cells and homologous chromosomal sequences." *Proc Natl Acad Sci U S A* **82(5)**: 1391-1395.
- Liu, W., Y. Xiong, et al.** (2006). "Stability and homogeneity of transgene expression in isogenic cells." *J Mol Med* **84(1)**: 57-64.
- Loew, R., Y. Meyer, et al.** "A new PG13-based packaging cell line for stable production of clinical-grade self-inactivating gamma-retroviral vectors using targeted integration." *Gene Ther* **17(2)**: 272-80.
- Logie, C. and A. F. Stewart** (1995). "Ligand-regulated site-specific recombination." *Proc Natl Acad Sci U S A* **92(13)**: 5940-4.
- Loonstra, A., M. Vooijs, et al.** (2001). "Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells." *Proc Natl Acad Sci U S A* **98(16)**: 9209-14.
- Lyznik, L. A., J. C. Mitchell, et al.** (1993). "Activity of yeast FLP recombinase in maize and rice protoplasts." *Nucleic Acids Res* **21(4)**: 969-75.
- McLeod, M., S. Craft, et al.** (1986). "Identification of the crossover site during FLP-mediated recombination in the *Saccharomyces cerevisiae* plasmid 2 microns circle." *Mol Cell Biol* **6(10)**: 3357-67.
- Meaking, W. S., J. Edgerton, et al.** (1995). "Electroporation-induced damage in mammalian cell DNA." *Biochim Biophys Acta* **1264(3)**: 357-62.

- Metzger, D. and P. Chambon** (2001). "Site- and time-specific gene targeting in the mouse." *Methods* **24**(1): 71-80.
- Metzger, D., J. Clifford, et al.** (1995). "Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase." *Proc Natl Acad Sci U S A* **92**(15): 6991-5.
- Mirabella, R., C. Franken, et al.** (2004). "Use of the fluorescent timer DsRED-E5 as reporter to monitor dynamics of gene activity in plants." *Plant Physiol* **135**(4): 1879-87.
- Missirlis, P. I., D. E. Smailus, et al.** (2006). "A high-throughput screen identifying sequence and promiscuity characteristics of the loxP spacer region in Cre-mediated recombination." *BMC Genomics* **7**: 73.
- Mlynarova, L., J. Libantova, et al.** (2002). "The promiscuity of heterospecific lox sites increases dramatically in the presence of palindromic DNA." *Gene* **296**(1-2): 129-37.
- Montoliu, L., S. Chavez, et al.** (2000). "Variegation associated with lacZ in transgenic animals: a warning note." *Transgenic Res* **9**(3): 237-9.
- Nagy, A.** (2000). "Cre recombinase: the universal reagent for genome tailoring." *Genesis* **26**(2): 99-109.
- O'Gorman, S., D. T. Fox, et al.** (1991). "Recombinase-mediated gene activation and site-specific integration in mammalian cells." *Science* **251**(4999): 1351-5.
- Orban, P. C., D. Chui, et al.** (1992). "Tissue- and site-specific DNA recombination in transgenic mice." *Proc Natl Acad Sci U S A* **89**(15): 6861-5.
- Oumard, A., J. Qiao, et al.** (2006). "Recommended Method for Chromosome Exploitation: RMCE-based Cassette-exchange Systems in Animal Cell Biotechnology." *Cytotechnology* **50**(1-3): 93-108.
- Palmiter, R. D., R. L. Brinster, et al.** (1982). "Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes." *Biotechnology* **24**: 429-33.
- Patsch, C., M. Peitz, et al.** "Engineering cell-permeant FLP recombinase for tightly controlled inducible and reversible overexpression in embryonic stem cells." *Stem Cells* **28**(5): 894-902.
- Peitz, M., K. Pfannkuche, et al.** (2002). "Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes." *Proc Natl Acad Sci U S A* **99**(7): 4489-94.
- Pham, C. T., D. M. MacIvor, B. A. Hug, J. W. Heusel, and T. J. Ley.** (1996). Long-range disruption of gene expression by a selectable marker cassette. *Proc Natl Acad Sci U S A* **93** (23):13090-13095
- Qiao, J.** (2009). "Systematic Study on Generation of Mammalian Production Cell Lines by Targeted Integration (RMCE)", Dissertation, TU-Braunschweig.

- Qiao, J., A. Oumard, et al.** (2009). "Novel tag-and-exchange (RMCE) strategies generate master cell clones with predictable and stable transgene expression properties." *J Mol Biol* **390**(4): 579-94.
- Rajewsky, K., H. Gu, et al.** (1996). "Conditional gene targeting." *J Clin Invest* **98**(3): 600-3.
- Raymond, C. S. and P. Soriano** (2007). "High-efficiency FLP and PhiC31 site-specific recombination in mammalian cells." *PLoS One* **2**(1): e162.
- Riu, E., D. Grimm, et al.** (2005). "Increased maintenance and persistence of transgenes by excision of expression cassettes from plasmid sequences in vivo." *Hum Gene Ther* **16**(5): 558-70.
- Roebroek, A. J., S. Reekmans, et al.** (2006). "Mutant Lrp1 knock-in mice generated by recombinase-mediated cassette exchange reveal differential importance of the NPXY motifs in the intracellular domain of LRP1 for normal fetal development." *Mol Cell Biol* **26**(2): 605-16.
- Roth, D. B. and J. H. Wilson** (1985). "Relative rates of homologous and non-homologous recombination in transfected DNA." *Proc Natl Acad Sci U S A* **82**(10): 3355-9.
- Rubsam, L. Z., P. D. Boucher, et al.** (1999). "Cytotoxicity and accumulation of ganciclovir triphosphate in bystander cells cocultured with herpes simplex virus type 1 thymidine kinase-expressing human glioblastoma cells." *Cancer Res* **59**(3): 669-75.
- Sadowski, P. D.** (1993). "Site-specific genetic recombination: hops, flips, and flops." *Faseb J* **7**(9): 760-7.
- Sandhu, U., M. Cebula, et al.** (2010). "Strict control of transgene expression in a mouse model for sensitive biological applications based on RMCE compatible ES cells." *Nucl. Acids Res.* Published online doi:10.1093/nar/gkq868
- Sauer, B.** (1996). "Multiplex Cre/lox recombination permits selective site-specific DNA targeting to both a natural and an engineered site in the yeast genome." *Nucleic Acids Res* **24**(23): 4608-13.
- Sauer, B. and N. Henderson** (1988). "Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1." *Proc Natl Acad Sci U S A* **85**(14): 5166-70.
- Sauer, B. and N. Henderson** (1989). "Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome." *Nucleic Acids Res* **17**(1): 147-61.
- Schebelle, L., C. Wolf, et al.** (2010) "Efficient conditional and promoter-specific in vivo expression of cDNAs of choice by taking advantage of recombinase-mediated cassette exchange using FIEEx gene traps." *Nucleic Acids Res* **38**(9): e106.
- Schlake, T. and J. Bode** (1994). "Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci." *Biochemistry* **33**(43): 12746-51.

- Schmidt, E. E., D. S. Taylor, et al.** (2000). "Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids." *Proc Natl Acad Sci U S A* **97**(25): 13702-7.
- Schnutgen, F., N. Doerflinger, et al.** (2003). "A directional strategy for monitoring Cre-mediated recombination at the cellular level in the mouse." *Nat Biotechnol* **21**(5): 562-5.
- Schnutgen, F., A. F. Stewart, et al.** (2006). "Engineering embryonic stem cells with recombinase systems." *Methods Enzymol* **420**: 100-36.
- Schubeler, D., K. Maass, et al.** (1998). "Retargeting of retroviral integration sites for the predictable expression of transgenes and the analysis of cis-acting sequences." *Biochemistry* **37**(34): 11907-14.
- Seibler, J.** (1999) "Sequenzspezifische Rekombination des Mammaliagenoms", Dissertation, Technische Universität Braunschweig.
- Seibler, J. and J. Bode** (1997). "Double-reciprocal crossover mediated by FLP-recombinase: a concept and an assay." *Biochemistry* **36**(7): 1740-7.
- Seibler, J., D. Schubeler, et al.** (1998). "DNA cassette exchange in ES cells mediated by Flp recombinase: an efficient strategy for repeated modification of tagged loci by marker-free constructs." *Biochemistry* **37**(18): 6229-34.
- Semprini, S., T. J. Troup, et al.** (2007). "Cryptic loxP sites in mammalian genomes: genome-wide distribution and relevance for the efficiency of BAC/PAC recombineering techniques." *Nucleic Acids Res* **35**(5): 1402-10.
- Senecoff, J. F. and M. M. Cox** (1986). "Directionality in FLP protein-promoted site-specific recombination is mediated by DNA-DNA pairing." *J Biol Chem* **261**(16): 7380-6.
- Shaikh, A. C. and P. D. Sadowski** (1997). "The Cre recombinase cleaves the lox site in trans." *J Biol Chem* **272**(9): 5695-702.
- Siegel, R. W., R. Jain, et al.** (2001). "Using an in vivo phagemid system to identify non-compatible loxP sequences." *FEBS Lett* **505**(3): 467-73.
- Smith, M. C. and H. M. Thorpe** (2002). "Diversity in the serine recombinases." *Mol Microbiol* **44**(2): 299-307.
- Smithies, O., R. G. Gregg, et al.** (1985). "Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination." *Nature* **317**(6034): 230-4.
- Stark, W. M., M. R. Boocock, et al.** (1992). "Catalysis by site-specific recombinases." *Trends Genet* **8**(12): 432-9.
- Steuber-Buchberger, P., W. Wurst, et al.** (2008). "Simultaneous Cre-mediated conditional knockdown of two genes in mice." *Genesis* **46**(3): 144-51.
- Takahashi, K. and S. Yamanaka** (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." *Cell* **126**(4): 663-76.

- Thomas, K. R. and M. R. Capecchi** (1987). "Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells." *Cell* **51(3)**: 503-12.
- Thyagarajan, B., M. J. Guimaraes, et al.** (2000). "Mammalian genomes contain active recombinase recognition sites." *Gene* **244(1-2)**: 47-54.
- Townes, T. M., J. B. Lingrel, et al.** (1985). "Erythroid-specific expression of human beta-globin genes in transgenic mice." *Embo J* **4(7)**: 1715-23.
- Turan, S.** (2007) "Charakterisierung mutierter FRT-Sites zum Austausch von Expressionskassetten", diploma thesis, Technische Universität Braunschweig.
- Umlauf, S. W. and M. M. Cox** (1988). "The functional significance of DNA sequence structure in a site-specific genetic recombination reaction." *Embo J* **7(6)**: 1845-52.
- Voelkel, C., M. Galla, et al.** "Protein transduction from retroviral Gag precursors." *Proc Natl Acad Sci U S A* **107(17)**: 7805-10.
- Volkert, F. C., D. W. Wilson, et al.** (1989). "Deoxyribonucleic acid plasmids in yeasts." *Microbiol Rev* **53(3)**: 299-317.
- Vooijs, M., J. Jonkers, et al.** (2001). "A highly efficient ligand-regulated Cre recombinase mouse line shows that LoxP recombination is position dependent." *EMBO Rep* **2(4)**: 292-7.
- Wodarczyk, C.** (2003) „Etablierung von ES-Zelllinien zur vorhersagbaren autoregulierten Expression von Transgenen in Mäusen“, Dissertation, Technische Universität Braunschweig
- Wong, E. T., J. L. Kolman, et al.** (2005). "Reproducible doxycycline-inducible transgene expression at specific loci generated by Cre-recombinase mediated cassette exchange." *Nucleic Acids Res* **33(17)**: e147.
- Wu, H., X. Liu, et al.** (1994). "Double replacement: strategy for efficient introduction of subtle mutations into the murine Col1a-1 gene by homologous recombination in embryonic stem cells." *Proc Natl Acad Sci U S A* **91(7)**: 2819-23.
- Yanez, R. J. and A. C. Porter** (1998). "Therapeutic gene targeting." *Gene Ther* **5(2)**: 149-59.
- Zahn-Zabal, M., M. Kobr, et al.** (2001). "Development of stable cell lines for production or regulated expression using matrix attachment regions." *J Biotechnol* **87(1)**: 29-42.
- Zufferey, R., T. Dull, et al.** (1998). "Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery." *J Virol* **72(12)**: 9873-80.

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Erklärung

Hiermit erkläre ich, dass ich die Dissertation (Versatile Extensions of the Flp-Recombinase-Mediated Cassette Exchange Technology: RMCE multiplexing approaches meet the needs for predictable genome engineering) selbstständig verfasst habe. Bei der Anfertigung wurden folgende Hilfen Dritter in Anspruch genommen (namentliche Nennung weiterer an der Dissertation beteiligter Personen und ihre Funktion bei der Erstellung der Dissertation):

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