

Yeast-based strategy for identification of 11 β -HSD1 inhibitors

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Abbreviations:

11 β -HSD1	11 β -Hydroxysteroid Dehydrogenase type 1
11 β -HSD2	11 β -Hydroxysteroid Dehydrogenase type 2
11 β -HSDs	11 β -Hydroxysteroid Dehydrogenases
bAS	β -Amirin Synthase
CAS	Cycloartenol Synthase
CBX	Carbenoxolone
cDNA	complementary Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide
DXM	Dexamethasone
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorting
FPP	Farnesyl Pyrophosphate
GC	Glucocorticoid
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
H6PDH	Hexose-6-Phosphate Dehydrogenase
LAS	Lanosterol Synthase
LUS	Lupeol Synthase
NADP ⁺	Nicotinamide Adenine Dinucleotide Phosphate
OSC	Oxidosqualene cyclase
TEV	Tobacco Etch Virus
TIPI	TEV protease Induced Protein Instability
UGT	Uridine diphosphate dependent Glycosyltransferases
yEGFP	yeast Enhanced Green Fluorescent Protein

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Abstract

Glucocorticoids are hormones with a vital role in regulation of metabolic and defence responses. Their metabolism plays an important role in the pathogenesis of obesity, a major risk factor for metabolic syndrome, a collection of disorders such as insulin resistance, dyslipidemia and hypertension.

At intracellular level the production of active glucocorticoids is regulated by two hydroxysteroid dehydrogenase enzyme isoforms 11 β -HSD1 and 11 β -HSD2, respectively. 11 β -HSD2 is predominantly expressed in mineralocorticoid target tissues and acts as oxidase catalysing conversion of cortisol into cortisone. The isoform 11 β -HSD1, expressed in a wide array of tissues and with highest levels in the liver, acts mainly as a reductase converting the cortisone into the biologically active hormone cortisol, in a NADPH dependent manner. Due to its implication in the metabolism of glucocorticoids, 11 β -HSD1 has become a primary target for the treatment of metabolic syndrome.

In the last years many strategies have been developed for the screening of molecules with inhibitory effects against this target enzyme. All known approaches present unique features and are suitable for specific screening models. Nevertheless, due to the great therapeutic and economical interest around the treatment of metabolic syndrome, many research groups are constantly focused on the development of new and more successful strategies for identification of hits with improved pharmaceutical properties.

In this study an innovative synthetic biology platform for synthesis and screening of 11 β -HSD1 inhibitors has been conceived and investigated. This strategy is based on the production of diverse chemical scaffolds in the yeast *Saccharomyces cerevisiae*, in which the molecules are directly screened through intracellular functional assays.

Providing all necessary genetic information, the biosynthetic pathway of plant triterpenoids, a big class of natural compounds with many beneficial effects on human health, was reconstructed in the baker's yeast. Moreover, by applying DNA family shuffling methods, a library of chimeric triterpenoid synthase cDNAs was constructed. The chimeric sequences, potential carriers of new enzymatic functions, were assembled together with the wild-type molecules in expressible yeast artificial chromosomes (eYACs).

Furthermore, the construction of two yeast assays strains functionally co-expressing the murine glucocorticoid receptor (GR) and the human 11 β -HSD1 enzyme is presented. In both developed strains, with different mechanisms, the reductase activity of the enzyme 11 β -HSD1 on cortisone is connected with an easily detectable fluorescent signal. Cells that produce compounds with inhibitory activity against 11 β -HSD1 can be identified through changes in fluorescence and isolated to further investigate the active molecules. The developed yeast-assays were validated using carbenoxolone, a

known 11 β -HSD1 inhibitor, and may represent useful tools for a first quick and easy screening of large number of membrane permeable putative inhibitory compounds.

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Introduction

Metabolic syndrome

Metabolic syndrome or syndrome X is a constellation of associated conditions such as increased blood pressure, high blood sugar level, excess body fat around the waist and abnormal cholesterol levels that occur together, increasing the risk of heart diseases, stroke and diabetes. In the last years, due mainly to the sedentary life style, the increasing prevalence of these conditions together with progression of obesity and diabetes around the world, have made metabolic syndrome an important public health concern (Levesque and Lamarche, 2008).

Metabolic abnormalities found in diabetes and metabolic syndrome are similar to those observed in the syndrome of prolonged glucocorticoid excess in blood (Cushing's syndrome) (Day, 2007). In Cushing's syndrome high production and high circulating levels of cortisol cause impaired glucose metabolism, decreasing glucose uptake in the peripheral tissues, enhancing the glucose production in the liver and decreasing the insulin production from pancreatic β -cells (Stahn et al., 2007).

Despite the high similarity of clinical conditions between metabolic and Cushing syndromes, obese or metabolic syndrome patients have normal or lower glucocorticoids levels in blood compared with patients suffering of Cushing's syndrome. This aspect led to the understanding that a tissue-specific peripheral activation process of these steroid hormones might be involved in pathogenesis of the conditions characterizing metabolic syndrome.

Glucocorticoid hormones

Glucocorticoids and mineralocorticoids are essential endocrine hormones involved in the regulation of many physiological processes. Mineralocorticoids, such as aldosterone in humans, principally stimulate epithelial sodium transport and regulate blood pressure (Agarwal and Mirshahi, 1999).

Glucocorticoids (GCs) are named on their primary role in glucose metabolism. They promote gluconeogenesis in the liver and oppose the action of insulin by directly inhibiting β -cells insulin secretion in the pancreas. They also have important functions in amino acids metabolism, immune system and adaptive response to physical and psychological stress (Baxter, 1974; Rhen and Cidlowski, 2005).

Both, glucocorticoid and mineralocorticoid hormones exert their functions upon binding to intracellular receptors, glucocorticoid and mineralocorticoid receptor respectively, with subsequent stimulation or repression of target genes transcription.

The biological activity of glucocorticoids relies on the presence of a hydroxyl group at position C-11 of the steroid structure. Cortisol in human and corticosterone in rodents are active steroids whereas cortisone and 11-dehydrocorticosterone, possessing a C-11 cheto group, are inactive molecules.

In humans, cortisol and part of cortisone are released under dynamic circadian and ultradian regulation by the hypothalamic-pituitary-adrenal axis and their rise in blood levels inhibits further release in a classic endocrine negative feedback loop (Biddie et al.2012). Cortisol is secreted in relatively high amount (15mg/day), but more than 90% of circulating hormone is bound to alpha 2-globulin, a cortisol binding globulin (Cope and Black, 1958; Esteban et al., 1991). Only free cortisol is biologically active and can be converted into cortisone in peripheral tissues (Andersen, 2002).

While cortisol is mainly secreted from the adrenal gland, the level of circulating cortisone in human body depends mainly on the peripheral conversion of cortisol (Tortorella et al., 1999). The low affinity between cortisone and alpha 2-globulin ensures similar circulating levels of free cortisone and free cortisol, despite the lower level of secreted cortisone (Meulenberg and Hofman, 1990).

The local metabolism of glucocorticoids is regulated by the enzymes 11 β -HSD1 and 11 β -HSD2 that modulate the intracellular availability of cortisol for the binding to glucocorticoid receptor (GR). The receptor in its unloaded form resides in the cytoplasm bound to chaperone proteins (Funder, 1996), then upon ligand binding, GR undergoes a conformational change and after dimerization translocates into the nucleus where it can exert its trans-activation and trans-repression actions.

Diversity in GR signaling is determined by the action of different glucocorticoid-response elements (GREs) and multiple receptor isoforms generated by alternative splicing and alternative translation initiation (Oakley and Cidlowski, 2011). Additionally, multiple post-translational modifications including phosphorylation, acetylation, ubiquitination, and SUMOylation can alter the function of this transcription factor (Anbalagan et al., 2012).

11 β -HSD enzymes

As described for the first time several decades ago, at a tissue-specific level the interconversion of glucocorticoids depends on the 11 β -HSD enzymes (Berliner and Dougherty, 1961; Atanasov and Odermatt, 2007). 11 β -HSD1 and 11 β -HSD2 act as chemical switches converting the active hormone cortisol into the inert cortisone and vice versa, regulating the intracellular availability of cortisol to the glucocorticoid receptor.

11 β -HSD1 and 11 β -HSD2 are products of separate genes and have different tissue distributions and physiological roles. The tridimensional structures of these two enzymes are similar with structurally conserved nucleotide cofactor binding and active sites. Nevertheless they share just the 18% of overall identity between their amino acid sequences (Tsigelny and Baker, 1995; Chapman et al., 2003).

11 β -HSDs belong to the short-chain dehydrogenases/reductases family of enzymes also known as short-chain alcohol dehydrogenases or secondary alcohol dehydrogenases (Nordling et al., 2002; Baker, 1996). The two isoforms seem to act in an antagonist manner: type 1 acts mainly as an 11-oxo reductase producing cortisol, while type 2 acts exclusively as a 11-hydroxyl dehydrogenase inactivating cortisol into cortisone (**Fig. 2.1**).

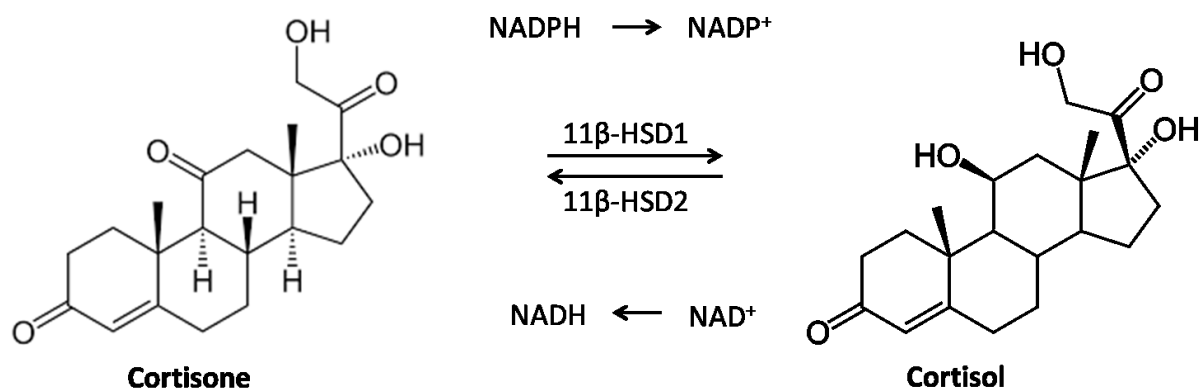


Figure 2.1. Reactions catalysed by 11 β -HSD enzymes.

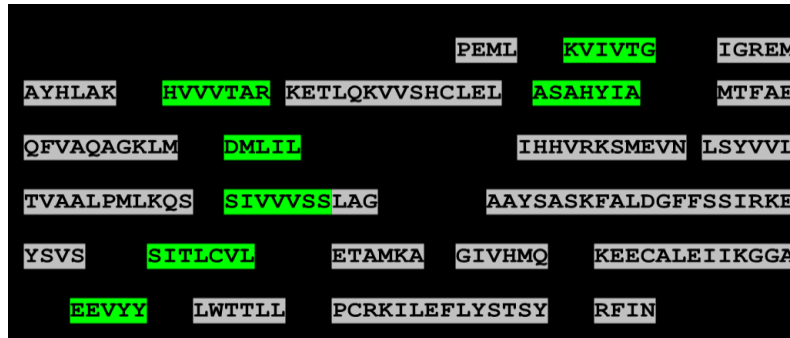
11 β -HSD2 is expressed predominantly in mineralocorticoid target tissues, such as salivary glands, colon and kidneys. Its role is to protect the nonselective mineralocorticoid receptor from the unspecific binding of cortisol. Impaired activity of 11 β -HSD2 causes the “apparent mineralocorticoid excess syndrome” due to the over activation of the mineralocorticoid receptor upon cortisol binding (Mune et al., 1995). 11 β -HSD2 is also highly expressed in the placenta and developing fetus, providing a potent barrier to maternal glucocorticoids (Seckl and Meaney, 2004). This isoform is a NAD⁺ dependent dehydrogenase and has a K_m in the nanomolar range for glucocorticoids (Albiston et al., 1994; Escher et al., 1998). Localization studies demonstrate that the bulk of the 11 β -HSD2 faces the cytosol and the N-terminal tail anchors the enzyme to the membrane of the endoplasmic reticulum (Naray and Fejes, 1996).

The isoform 11 β -HSD1 is found as homodimer (Zhang et al., 2005; Hosfield et al., 2005) and is expressed in a wide array of tissues, with highest levels in the liver, from where it was originally purified (Lakshmi and Monder, 1988). The encoding human gene, *HSD11B1*, is over 30 kb in length and consists of 6 exons localized on chromosome 1 (1q32.2–41). A cDNA of approximately 1.4 kb in length predicts an open reading frame of 876 bp and a protein of 292 amino acids (**Fig. 2.2a**). Expression in human is absent until 2–3 months of age, then rises until 1 year of age, when it reaches constant levels.

11 β -HSD1 structure can be divided into four important regions. The transmembrane domain enabling the enzyme to attach the membrane of ER with a short five amino acid region in the cytosolic side (Odermatt et al., 1999), the cofactor binding domain (Rossmann fold), the catalytic site, and the C-terminal domain involved in the oligomerisation of the enzyme (**Fig. 2.2b**).

Ser170, Tyr183, Lys187 and Asn111 are conserved residues of the catalytic site responsible for the substrate orientation, proton transfer and reaction intermediates stabilization (Filling, 2002; Hosfield et al., 2005). The enzyme has a K_m in the range of μ M for its glucocorticoid substrates and shows preference in binding cortisone rather than cortisol supporting the fact that *in vivo* it acts primarily on the cortisone as a reductase (Tannin et al., 1991; Stewart et al., 1994).

a.



b.



Figure 2.2. a) Amino acid sequence of 11 β -HSD1 human enzyme. In bold are indicated the amino acids involved in the secondary conformation of the enzyme. Grey indicates helix and green indicates beta strands.

b) Tridimensional structure of human 11 β -HSD1 homodimer. Red arrows indicate the amino-terminal end of each monomer. Green arrow indicates the C-terminus of the two monomers interacting in the homodimer complex (Protein Data Bank archive).

Enzymatic studies of the liver 11 β -HSD1 underlined a bidirectional conversion of GCs. This enzyme is able in fact to reduce cortisone into active cortisol with NADPH cofactor as source of protons, or to act as dehydrogenase on cortisol converting it to cortisone. Upon cell disruption the reductase activity of 11 β -HSD1 is lost (Lakshmi and Monder, 1988; Duperrex et al., 1993; Low et al., 1994) and can be regained providing high levels of NADPH cofactor in the reaction environment (Walker et al., 2001).

From recent observations it is clear that the enzymological characteristics of 11 β -HSD1 critically depend on its intracellular compartmentalization. The N-terminal tail of the enzyme anchors it to the endoplasmic reticulum (ER) membrane with the catalytic domain facing the lumen of the ER. Within the N-terminal tail two positively charged lysine residues on the cytoplasmic side and two negatively

charged glutamate residues in the luminal side are crucial for the correct orientation of the enzyme in the ER membrane (Frick et al., 2004). Exchange of N-terminal regions between the 11 β -HSD1 and 11 β -HSD2 led to inverted orientation within the ER and both chimeric proteins resulted inactive (Odermatt et al., 1999).

In the lumen of ER the enzyme 11 β -HSD1 co-localizes and functionally depends from the hexose-6-phosphate dehydrogenase (H6PDH), the microsomal isoform of the well-known glucose-6-phosphate dehydrogenase. H6PDH converts glucose-6-phosphate to 6 phosphogluconolactone generating the reduced cofactor NADPH (Atanasov et al., 2004; Bujalska et al., 2005) (**Fig. 2.3**).

This enzymatic collaboration was finally confirmed through knock out mice lacking the *H6PDH* gene in which no conversion of the inactive glucocorticoid into corticosterone was detected (Lavery et al., 2006). A protein-protein interaction between the two enzymes allows a fine tuning regulation of the 11 β -HSD1 activity due to the direct supply of NADPH from H6PDH (Atanasov et al., 2008).

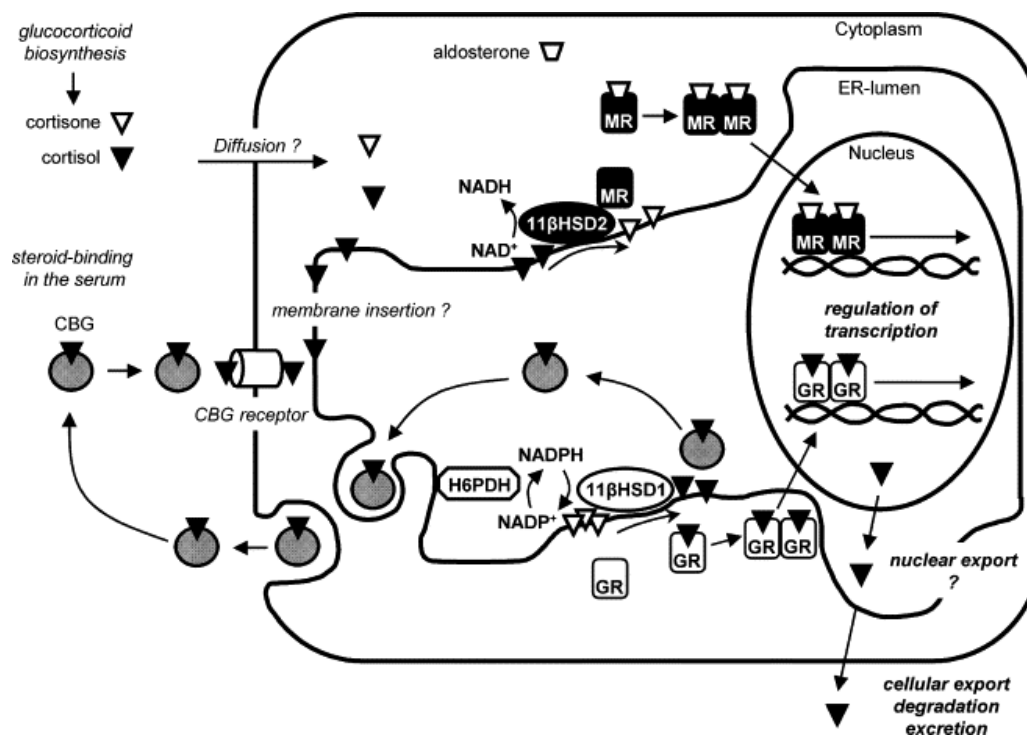


Figure 2.3. Schematic representation of glucocorticoids action mediated by 11 β -HSD enzymes in human cells (Reproduced with permission from Odermatt et al., 2006).

11 β -HSD1 and metabolic diseases

Implication of 11 β -HSD1 in the pathogenesis of metabolic diseases has been brought to light from investigations conducted on obese individuals that revealed a positive correlation between expression of this enzyme in subcutaneous adipose tissue and the percentage of body fat, waist circumference, fasting glucose and insulin resistance (Rask et al., 2002; Desbriere et al., 2006). Moreover, the importance of 11 β -HSD1 and high levels of cortisol in adipose tissues has been elegantly demonstrated in transgenic mice experiments (Paterson et al., 2005). Mice with 11 β -HSD1 overexpression simulate a condition very similar to the metabolic syndrome, with elevated local levels of cortisol that antagonize the effects of insulin contributing to the development of insulin resistance and type 2 diabetes mellitus (Masuzaki et al., 2001; Fotsch and Wang, 2008). On the contrary, 11 β -HSD1 knock out mice, are viable healthy models with adrenal hyperplasia and decreased production of active cortisol in the peripheral tissues. They showed a resistance to diet induced obesity and an improved lipid profile and glucose tolerance (Kotelevtsev et al., 1997; Morton et al., 2001). Similar results were obtained under disruption of 11 β -HSD1 in an obesity/diabetes prone mice strain (Morton et al., 2004).

Studies on animal models revealed the important role of 11 β -HSD1 in the peripheral metabolism of glucocorticoids and made of this enzyme the most relevant target for the treatment of metabolic syndrome and its associated disorders. In fact, the inhibition of the 11 β -HSD1 enzyme is a successful strategy for decreasing tissues cortisol accumulation and to treat the metabolic consequences of glucocorticoid receptor hyper-activation in peripheral tissues.

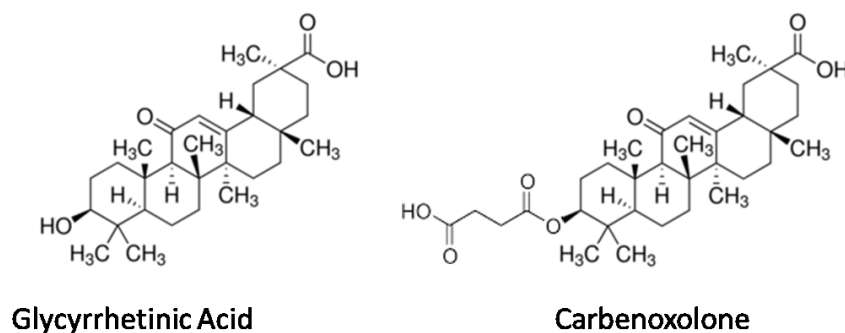
Inhibition of 11 β -HSD1

Many natural and synthetic compounds have been investigated for their ability to inhibit the enzyme 11 β -HSD1. One of the first described natural inhibitor is glycyrrhetic acid, a triterpenoid found in liquorice root (*Glycyrrhiza glabra*) and already used in traditional medicine for thousands of years thanks to its beneficial effect on human health (Monder et al., 1989) (**Fig. 2.4a**).

In the selection process of new putative inhibitors against the 11 β -HSD1 enzyme, different properties of the tested compounds, such as species-specific effects and specificity, have to be evaluated.

Since numerous *in vivo* pharmacological studies are carried out in rodents, it is necessary for inhibitors of human 11 β -HSD1 to also inhibit the rodent enzyme. The crystal structures of rodent and human 11 β -HSD1 are considerably different with a similarity of 77% at the amino acid level (Tannin et al., 1991). Therefore, a compound able to inhibit the human enzyme will probably not have the same effect and strength on the rodent enzyme. This is, for example, the case of arylsulfonamidothiazole compounds that show different behaviours towards 11 β -HSD1 enzymes found in different species. In contrast the carbenoxolone, hemisuccinyl ester of glycyrrhetic acid, is able to strongly bind and inhibit both the mouse and human enzymes with similar effects (Su et al., 2008) (Fig. 2.4b).

a.



b.

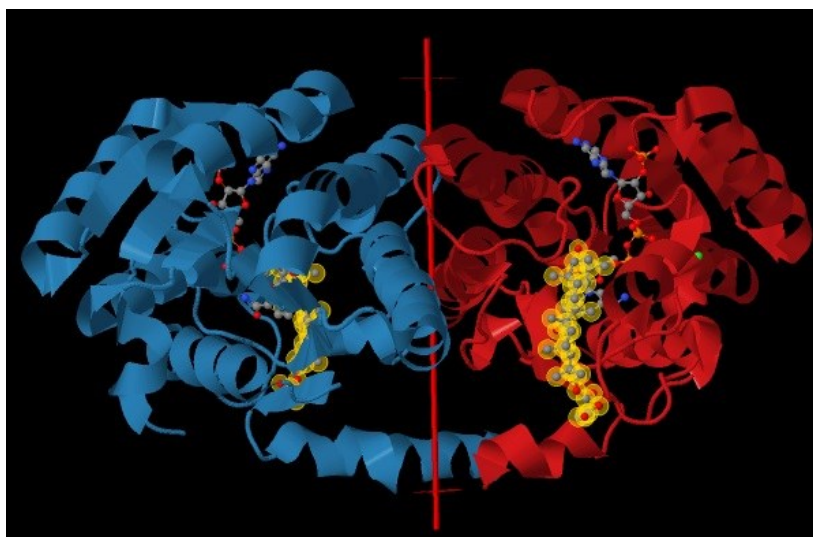


Figure 2.4. a) Chemical structures of two common 11 β -HSD1 inhibitors. b) Tridimensional structure of human 11 β -HSD1 homodimer complex. The two monomers (red and blue) interact with carbenoxolone (yellows halos) in the catalytic pocket (Protein Data Bank archive).

In the screening processes for new 11 β -HSD1 inhibitors complementary studies concentrate on the specificity of the candidate molecules, studying their effects towards others human enzymes (Arampatzis et al., 2005). In the case of 11 β -HSD1 inhibition, the typical direct association for a specificity control of a candidate molecule is to study its effects against the 11 β -HSD2 enzyme. In fact, as for glycyrrhetic acid and carbenoxolone, intensive therapeutic programs employing 11 β -HSD1 inhibitor with strong inhibitory effect also on the isoform 2 would cause important consequences as sodium retention, hypokalemia and hypertension (Kotelevtsev et al., 1999; Sandeep et al., 2004). However, the lack of specificity might affect also other enzymes with high similarity to 11 β -HSD1 such as retinol dehydrogenases and 17 β -HSD that, structurally related with 11 β -HSD1, if improperly inhibited would cause important health issues (Robinzon et al., 2003).

All known 11 β -HSD1 inhibitors can be divided in several groups: endogenous substances, exogenous natural products and their derivatives and synthetic small organic molecules.

Several endogenous steroids, including progesterone and its metabolites, androgen metabolites and many bile acids have been reported to act as 11 β -HSD1 inhibitors although their effect is not specific and rather weak (Morris et al., 2004). Among the natural occurring 11 β -HSD1 inhibitors, glycyrrhetic acid and its derivative carbenoxolone are the most used compounds for inhibition of 11 β -HSD1 in “proof of concept” experiments. Nevertheless, as already mentioned before, they lack of specificity with respect to the 11 β -HSD2 and, moreover, carbenoxolone seems to have limited ability to penetrate adipose tissue where the effect of the 11 β -HSD1 inhibition should be crucial to lighten insulin resistance (Sandeep et al., 2005). Flavonoids from fruits and vegetables are other non-selective 11 β -HSD1 inhibitors (Lee et al., 1996) and also coffee has been tested and some of its components showed a strong inhibitory effect and high specificity towards 11 β -HSD1 (Atanasov et al., 2006). In addition, many synthetic molecules like adamantly triazole and 2 aminothiazole sulfonamide have shown good inhibition and specificity against 11 β -HSD1 (Barf et al., 2002; Hermanowski-Vosatka et al., 2005).

Most of the approaches used for selection of candidate inhibitors employ liver microsomes or recombinant mammalian cell lines expressing 11 β -HSD1 as source of the enzyme and analyse cortisol and cortisone contents through Liquid Chromatography-Mass Spectrometry (Xu et al., 2006). Other strategies involve the stable heterologous expression of 11 β -HSD1 combined with β -galactosidase reporter constructs under control of glucocorticoid response elements (Barton et al., 2004).

Moreover, in-silico approaches, such as pharmacophore modelling, are extensively used. These methods generally study the electrostatic and steric features that are necessary for an optimal interaction of a small molecule with its biological target (enzyme, receptor) and allow for selection of

hits through the screening of huge virtual library of compounds (Schuster et al., 2006; Vuorinen et al., 2014).

All these strategies represent powerful tools to filter and select active compounds from large libraries of molecules. Nevertheless they often require the use of hazardous radioactive substances or the use of colorimetric materials that can interfere with the tested molecules and they can require expensive instrumentation and expertise. Therefore, due to the large economic and pharmaceutical interest around the inhibition of this target enzyme, many research groups are still focused on development of more efficient strategies that using innovative screening systems, enable to isolate molecules with improved therapeutic effects.

Triterpenoids

Among all the molecules investigated for the inhibition of 11 β -HSD1, natural compounds have attracted much attention. Historically, in ancient eastern medicine practices, many plant extracts were used for their important and relevant effects on human health. In fact, studies of roots and bulks extracts with antidiabetic properties has allowed for the identification of interesting natural compounds that affect the function of 11 β -HSD1 in the metabolism of glucocorticoids (Gumy et al., 2009). Many classes of natural compounds like flavonoids (Schweizer et al., 2003), plant polyketides and triterpenoids (Blum et al., 2009) have been described to have inhibitory effects on 11 β -HSD enzymes. Particularly triterpenoids have attracted high attention during the last years due to their wide applications in medicine with anti-inflammatory (Tapondjou et al., 2008; Sun et al., 2010), anti-cancerogenic (Man et al., 2010) anti-bacterial (Saleem et al., 2010) anti-fungal and antiviral effects (Coleman et al., 2010; Rattanathongkom et al., 2009).

Triterpenoids represent a large group of plant natural compounds with more than 20'000 known members. They are synthesized from the condensation of five-carbon building blocks of the mevalonate pathway in fifteen-carbon building block units of farnesyl pyrophosphate (FPP). Two FPP are then fused to form 2-3 oxidosqualene (Kirby and Keasling, 2009). The cyclization of the 30-carbon intermediate oxidosqualene, catalysed by oxidosqualene cyclases (OSCs) also known as triterpenoids synthases or cyclases, represents the first distinct step in the synthesis of triterpenoids (**Fig. 2.5**). This class of molecules includes sterols, as lanosterol and cycloartenol, steroids, and triterpenoid saponins. In general, animals and fungi have only one oxidosqualene cyclase, called lanosterol

synthase (LAS), needed for production of membrane sterols and steroids. In plants, cycloartenol synthase (CAS) is widely distributed but also LAS is represented in some species (Suzuki et al., 2006). These two enzymes convert oxidosqualene through the protosteyl cation intermediate in different triterpenoid backbones. Many other cyclases have evolved from common ancestral CAS (Lodeiro et al., 2005) and are able to synthesize different triterpenoid frameworks (Ohya et al., 2009; Xu et al., 2004).

Among the OSCs two of the more represented in plants are the lupeol synthases (LUS) and β -amyrin synthases (bAS). LUSs and bASs cyclize oxidosqualene through dammarenyl cation in order to form lupeol and β -amyrin, respectively. Lupeol, β -amyrin and their diverse metabolites are implicated in various plant processes and seem to act in plants as protective agents against parasites and insects (Augustin et al., 2011).

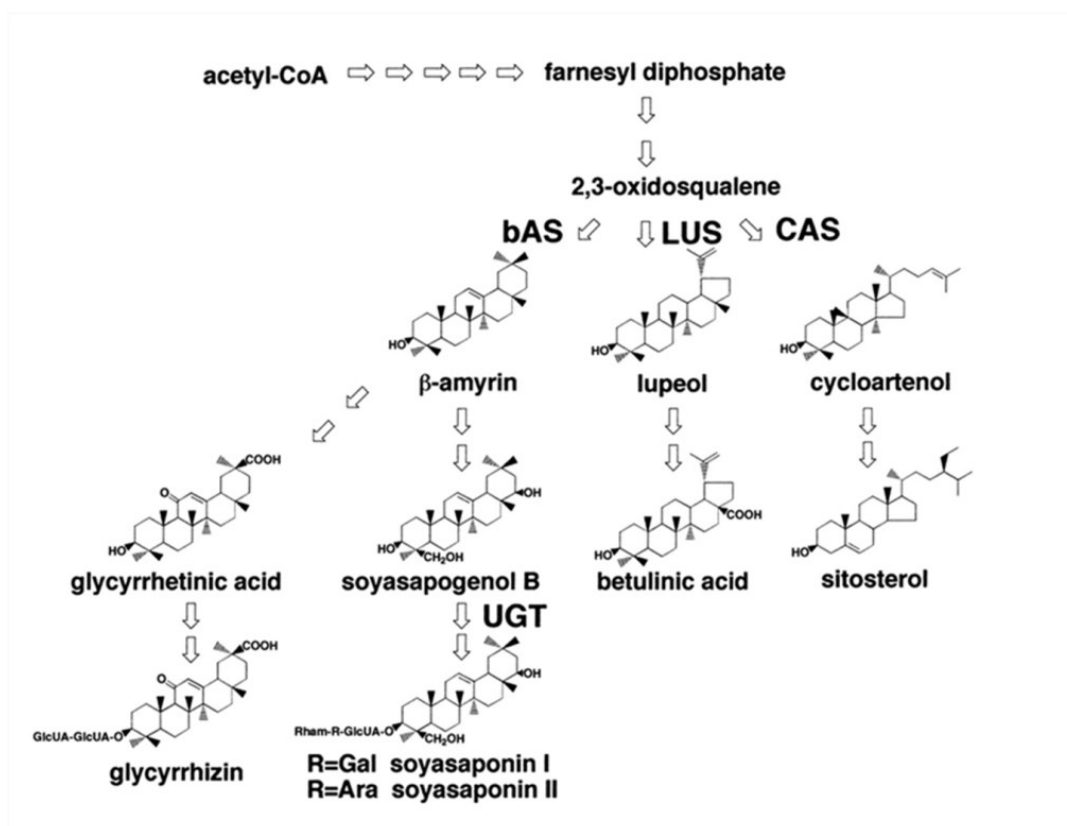


Figure 2.5. Schematic representation of the triterpenoids biosynthetic pathway. The most common triterpenoid backbones are represented. bAS= beta-amyrin synthase; LUS= lupeol synthase; CAS= cycloartenol synthase; UGT= uridine diphosphate glycosyltransferases (Reproduced with permission and adapted from Hayashi et al. 2003).

The diversity of plants triterpenes emanates not only from the radiation of the triterpenoid synthase genes family but also from the ability of some family members to contribute multiple products (Morita et al., 2000; Segura et al., 2000). The oxidosqualene cyclases that produce multiple products, classified as “multifunctional”, are not yet deeply understood from the enzymatic point of view. However, some researches have confirmed, through mutagenesis and directed evolution experiments, that evolution progresses are responsible for big changes in product diversity formation from a single parental enzyme (Segura et al., 2003).

In plants, after the cyclization of the 2-3 oxidosqualene into the basic triterpenoid backbones, the resulting molecules are always modified through reactions performed by P450 monooxygenases and uridine diphosphate dependent glycosyltransferases (UGTs) (Hamberger and Bak, 2013).

P450s act on specific substrates and allow the modification in a target position of the backbone. Due to the high number of P450s present in plants (1% of all protein encoded) many modifications as hydroxyl, keto-, aldehyde- and carboxyl- groups can be found in the final molecules. This first step of modification contributes to increase the number of different triterpenoids present in nature (Seki et al., 2015). The primary modification can also be followed by other decoration steps as the linkage of complex side groups or formation of intramolecular bridges between adjacent functional groups.

Glycosylation presumably involves sequential activity of different enzymes belonging to the multigene family of UGTs that catalyse the transfer of activated glycosyl residues from uridine sugar nucleotides to a vast array of acceptor molecules (Vogt and Jones, 2000; Ross et al., 2001). Once glycosylated, triterpenoid saponins increase their water solubility and acquire specific activity in the plant organism.

Among their many industrial applications, during the last decades high interest has been focused on selection or development of triterpenoid molecules with inhibitory effect on enzyme 11 β -HSD1. Glycyrrhetic acid and its derivative carbenoxolone have been found to have potent effects in the 11 β -HSD1 inhibition. Glycyrrhetic acid is a triterpenoid found mainly in roots of *Glycyrrhiza glabra* and derives from the triterpenoid backbone of β -amyrin. All the enzymes responsible for the synthesis of this triterpenoid have been studied through coupling of phytochemical approaches and analytical chemistry. β -amyrin synthase of *G.glabra* was identified in 2001 (Hayashi et al., 2001) and the P450s (CYP88D6 and CYP12A154) responsible for the modifications at position C-11 and C-30 of β -amyrin have been identified from their natural source and their activities were confirmed in yeast (Seki et al., 2008 and 2011) (**Fig. 2.6**). Currently, glycyrrhetic acid is used as important lead compound for designing of analogues with more specific inhibitory effects on the human enzyme 11 β -HSD1 (Su et al., 2007; Beseda et al., 2010).

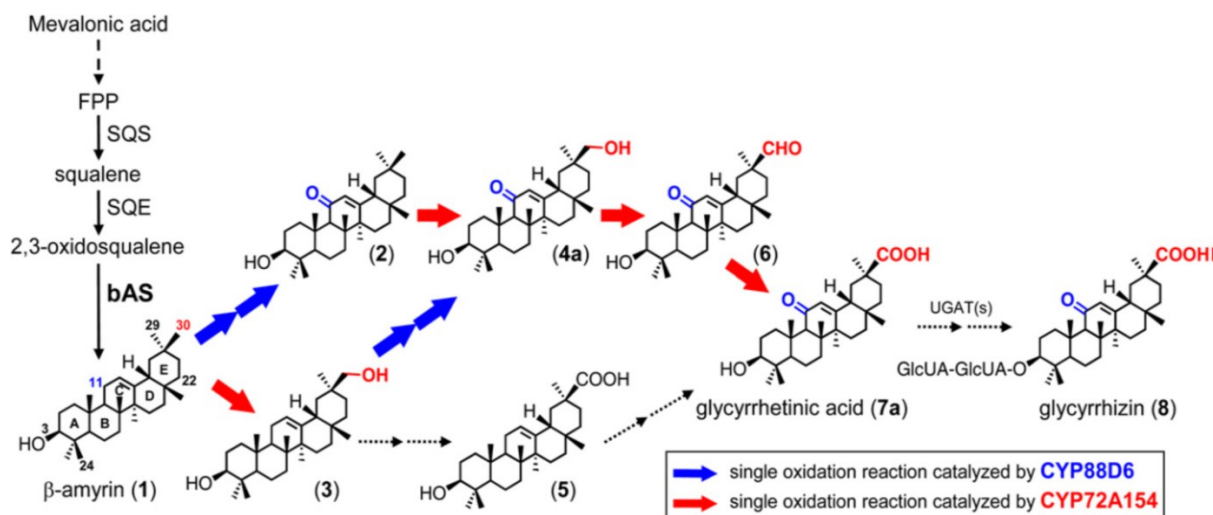


Figure 2.6. Proposed pathway for the synthesis of glycyrrhetic acid. The P450 enzymes CYP88D6 and CYP72A154 are responsible for the secondary modifications on the β -amyrin backbone (Reproduced with permission from Seki et al., 2011).

An additional reason of the large number of triterpenoids present in nature can be addressed to the evolutionarily plasticity of the triterpenoid synthases that readily change and acquire new catalytic properties (Trapp and Croteau, 2001). This feature has allowed this family of enzymes to expand and form high number of members displaying different catalytic features. Change in residues that sterically distort the catalytic domain of the enzyme could lead easily to a change in the final product of the cyclization processes. Application of random mutagenesis approaches such as DNA shuffling or random points mutagenesis have allowed changes in product specificity of triterpenoid synthases leading to the formation of different products with respect to the original ones (Wu and Griffin, 2002; Segura et al., 2003).

3

Aim of the study

In the last decades the great medical and economical interest around the treatment of metabolic syndrome and related disorders has been the propellant force leading to the identification of pharmaceutical compounds with inhibitory activity against the human enzyme 11β -HSD1. In fact, with its pivotal role in glucocorticoids metabolism, 11β -HSD1 represents the main pharmaceutical target for treatment of metabolic syndrome.

Screenings of chemical libraries and natural molecules represent the main sources of new inhibitors and are supported by valuable assay systems where the function of the target enzyme can be easily investigated. However, these traditional screening approaches usually explore just small fractions of the natural chemical diversity.

This work aims at the construction of a synthetic biology platform for the synthesis, screening and identification of 11β -HSD1 inhibitors. This strategy is based on the production of diverse chemical scaffolds in the yeast *Saccharomyces cerevisiae*, in which the molecules are directly screened through intracellular functional assays. The synthesis of novel compounds is based on reconstitution of biosynthetic pathways, known to be source of drug-relevant chemical scaffolds, in the host organism through providing the appropriate genetic information. In addition, the chemical variability synthesized can be further increased by providing genetic sequences with unknown functions that can reveal new enzymatic activities (Klein et al., 2014) (Fig.3.1).

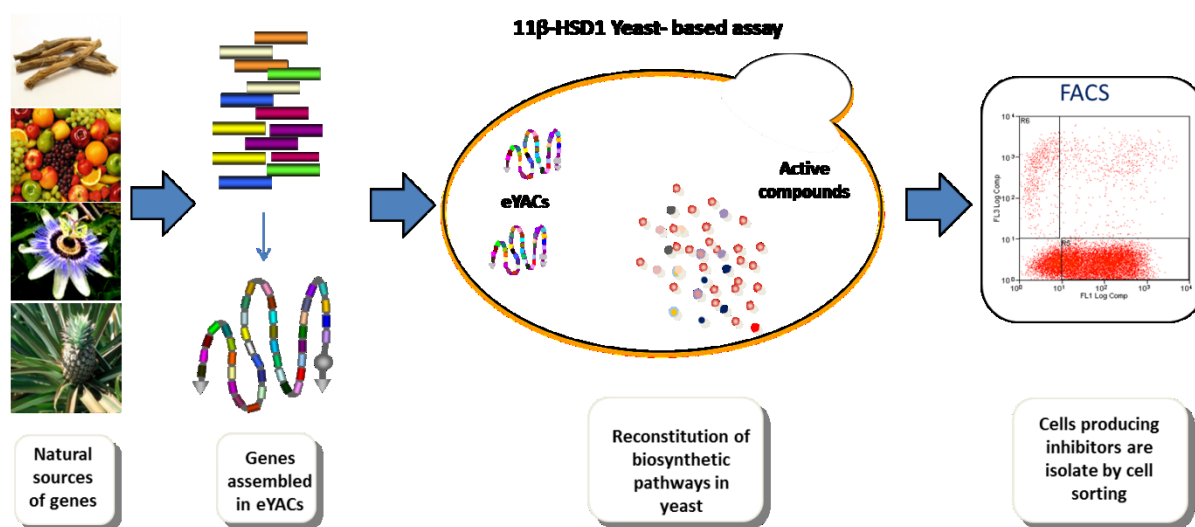


Figure 3.1. Schematic representation of the strategy investigated within this PhD thesis.

The application of the synthetic biology platform for synthesis and screening of human 11 β -HSD1 inhibitors can be divided into three sections:

1) Selection of genetic information for reproduction of natural drug-relevant pathways in yeast:

In this work, the biosynthetic pathway of triterpenoids was selected as source of drug-relevant genetic information. Triterpenoids are mainly represented in plants and belong to terpenoids, the biggest class of natural compounds. Several beneficial effects on human health have been attributed to these molecules and many representatives are currently used for prevention and treatment of different pathological conditions. Various components of this class have been demonstrated to have inhibitory effects on the 11 β -HSD1 enzyme, but generally, due to low target specificity, they cannot be used intensively in therapy. For these reasons further investigations within this class of metabolites represent a successful strategy for identification of more specific inhibitors of the target enzyme.

Natural synthesis of triterpenoids is an elaborated biochemical process that involves cyclization of squalene molecules into complex 30 carbons scaffolds that can be further modified by addition of functional chemical groups. The whole pathway is difficult to be reproduced through synthetic chemistry and in some cases not all steps involved are clearly defined. Therefore for many years the main source of these compounds was the extraction from their natural plant producers. However this step represented a bottle neck for the study of their pharmaceutical properties due to low yields and purity of the extracts. In order to overtake these limitations, strategies based on metabolic engineering of microorganisms represent valuable alternatives.

Here, the biosynthetic pathway leading to production of plant triterpenoids is reconstituted in yeast *Saccharomyces cerevisiae* by using eYACs (expressible Yeast Artificial Chromosomes). eYACs allow for the expression of large numbers of heterologous genes in yeast providing all the essential genetic information for the assembly of the biosynthetic pathway of triterpenoids.

Yeast cells are ideal host organisms for the synthesis of this class of natural compounds, given the fact that squalene and 2,3-epoxy-squalene, the common precursors to all triterpenoids, are produced in yeast as precursors of ergosterol. Therefore, the endogenous ergosterol pathway can be exploited for production of these plant metabolites, providing the yeast with the exogenous genetic information that leads from epoxy-squalene to synthesis of the triterpenoid backbones and their functionalized derivatives (**Fig. 3.2**).

2) Construction of new genetic information in order to increase the variability of compounds synthesized in yeast:

Trying to extend the variability of the chemical structures synthesized in the yeast cells, unknown genetic information are also sourced to the host organism. These information lead to the expression of new enzymes with distinct catalytic activities that can originate new chemical scaffolds or modify the pre-synthesized ones.

To this purpose, in this thesis, a group of cDNA molecules codifying for natural triterpenoid cyclases (β -amyrin-, lupeol-, and cycloartenol- synthases) is subjected to protein engineering methods. The resultant chimeric sequences are collected in a large chimeric cDNA library generated by DNA shuffling procedures, and included in the eYACs preparation. Reiterative cycles of shuffling has proved to be a useful approach for the evolution of single gene products with enhanced activity, altered substrate and product specificity (Cramer et al., 1998).

Finally, the genetic information provided by eYACs, including natural triterpenoid synthase cDNAs and mutant triterpenoid cyclase sequences, can be further increased with a collection of genes codifying for “decoration” enzymes (e.g. P450 oxygenases, glycosyltransferases etc.) that enable to modify the chemical scaffolds produced, dramatically enhancing their variability.

Altogether these elements aim to increase the diversity of compounds that can be synthesized in yeast and give access to a broad chemical diversity for screening of pharmaceutical active compounds against the enzyme 11 β -HSD1 (**Fig. 3.2**).

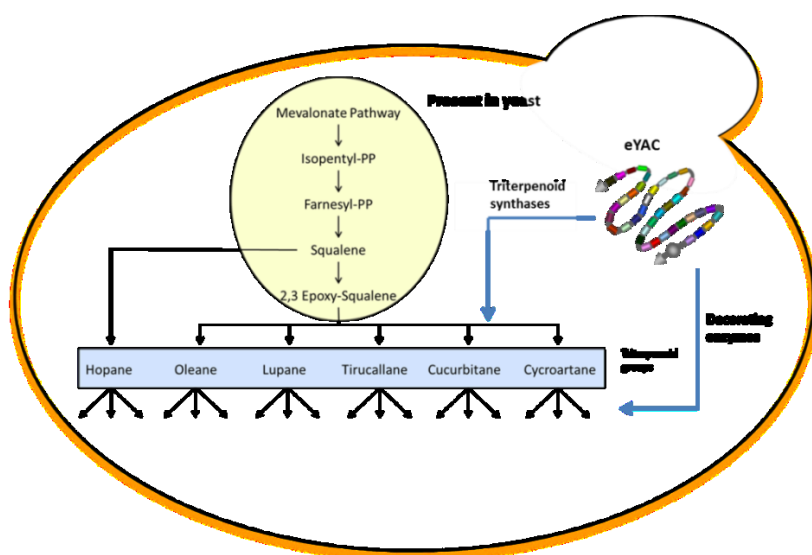


Figure 3.2. Reconstitution of the triterpenoid biosynthetic pathway in yeast using the eYAC approach. Natural and chimeric triterpenoid synthases are responsible for the conversion of squalene or 2,3 epoxy-squalene in different chemical scaffolds. Decorating enzymes can act on the pre-synthesized frameworks and modify them through addition of functional groups.

3) Construction of yeast based screening assays for identification of human 11 β -HSD1 inhibitors:

The synthetic biology platform investigated in this thesis is based on the potential of yeast as model organism. Yeast cells represent not only the producers of diverse chemical scaffolds, but in addition the screening systems for the effects of the new synthesized compounds versus the pharmaceutical target, the human enzyme 11 β -HSD1.

In order to screen the large collection of compounds that can be produced in yeast by the eYAC approach and to avoid long and complex purification processes, the molecules are meant to be directly tested at intracellular level through efficient yeast based assays in which the activity of 11- β HSD1 is connected with a fluorescent output. Molecules that exert inhibitory activity cause intracellular changes of fluorescence and the yeast cells producing inhibitor compounds can be identified and sorted by FACS.

This thesis in particular describes:

- **The construction of a large library of chimeric triterpenoid synthase cDNAs through application of DNA family shuffling procedures.**

- **The assembling and transformation of eYAC molecules for simultaneous expression of triterpenoid cyclases (natural and chimeric) and the investigation of their capacity for synthesis of triterpenoid molecules in yeast.**

- **The construction, testing and validation of two yeast assay strains where the activity of human enzyme 11 β -HSD1 is connected to a fluorescent output through different mechanisms.**

4

Results and Discussions

Evolutionary methods

*In the present chapter the study and the application of DNA evolutionary strategies to three groups of natural triterpenoid synthase cDNAs is described. The new DNA chimeric molecules, together with cDNAs of natural triterpenoid synthases and “decorating” enzymes, were aimed to be assembled in eYACs for multiple and simultaneous expression in the yeast *Saccharomyces cerevisiae*. Yeast cells provided with these genetic information contain all the essential elements for synthesis of plant triterpenoids and presumably other unknown chemical scaffolds to be screened for 11 β -HSD1 inhibition.*

Despite continued advances in the understanding of enzymes structures and functions, it is clear that many aspects of enzyme activity cannot be predicted. It is for this reason that strategies for protein engineering attract large consensus.

Evolutionary methods in biology are potent strategies for the *in vitro* evolution of enzymes and tend to mimic the natural process of evolution speeding it up by direct *in vitro* selection. The most successful and applied techniques rely on heterologous recombination of fragments from related natural genes for construction of large libraries of new chimeric molecules. These strategies, based on fragments recombination, allow to investigate the functions of amino acid domains or to randomly introduce modifications in the new mutant enzymes.

Generally, for the application of these protocols not deep information on the enzymes activity are needed and the large amount of chimeric sequences produced represents a promising source for isolation of new interesting enzyme variants. In fact, it has been extensively demonstrated that properties such as productivity, substrate and product specificities or reaction mechanism of an enzyme can be changed dramatically through application of these methods (Stemmer, 1994; Cramer et al., 1998; Zhang et al., 1999; Ness et al., 1999).

In order to create a large cDNA chimeric library searching for new enzymatic activities, ten cDNA molecules, codifying for natural triterpenoid synthases, have been selected for the application of two DNA shuffling methods. The selected molecules codify for three of the most common classes of triterpenoid cyclases present in plants: cycloartenol-, β -amyrin- and lupeol- synthases (**Table 4.1**). The expression and functionality of these classes of enzymes have been previously described in yeast (Corey et al., 1993; Herrera et al., 1998; Iturbe-Ormaetxe et al., 2003).

Table 4.1. Triterpenoid cyclase cDNAs used in the application of DNA shuffling protocols.

cDNA	Name	Abbreviation	Source	Accession number
EV42	Lupeol Synthase	LUS	<i>Arabidopsis thaliana</i>	NM_179572
EV43	Lupeol Synthase	LUS	<i>Ricinus communis</i>	DQ268869
EV47	β -amyrin synthase	bAS	<i>Medicago truncatula</i>	AF478453
EV48	β -amyrin synthase	bAS	<i>Glycyrrhiza glabra</i>	AB037203
EV49	β -amyrin synthase	bAS	<i>Panax ginseng</i>	AB009030
EV50	β -amyrin synthase	bAS	<i>Panax ginseng</i>	AB014057
EV52	Cycloartenol synthase	CAS	<i>Lotus japonicus</i>	AB181246
EV53	Cycloartenol synthase	CAS	<i>Glycyrrhiza glabra</i>	AB025968
EV54	Cycloartenol synthase	CAS	<i>Panax ginseng</i>	AB009029
C405	Cycloartenol synthase	CAS	<i>Pisum sativum</i>	D89619

Triterpenoid synthase enzymes have a high evolutionary plasticity and readily change acquiring new catalytic properties (Trapp and Croteau, 2001). Studies on their evolution have suggested that they all derived from a common ancestral molecule through an intense evolutionary process (Xue et al., 2012). Cycloartenol synthases (CAS) are considered the common ancestral oxidosqualene cyclases and have proven to be excellent targets for mutagenesis, easily acquiring new catalytic properties (Wu and Griffin, 2002; Meyer et al., 2002). They were the first plant triterpene synthases identified through complementation experiment in a lanosterol synthase mutant yeast (Corey et al., 1994).

β -amyrin and lupeol synthases represent a distinct catalytic class with respect to cycloartenol synthases and produce non-steroidal triterpenoids. These cyclases arose recently and are only represented in dicotyledonous plants (Segura et al., 2003). Their amino acid sequences generally share high similarity despite generating different products. Random mutagenesis studies demonstrated high plasticity of these enzymes that efficiently interchanged their product specificity (Shibuya et al., 1999).

Moreover, β -amyrin cyclases (bAS) catalyse the synthesis of β -amyrin, precursor of glycherretinic acid, one of the most studied inhibitors of 11 β -HSD1 (Seki et al., 2011). Random mutagenesis of this enzyme might represent a promising strategy to generate changes in its reaction mechanism in order to produce new interesting chemical scaffolds with stronger and more specific inhibitory effect against 11 β -HSD1.

Different works, through mutagenesis approaches, have demonstrated the flexibility of these enzymes in switching their catalytic properties, for example conversion of a cycloartenol synthase into a lanosterol synthase (Wu and Griffin, 2002). However, no mutations of triterpenoid synthases that lead to synthesis of novel and new-to-nature triterpenoid scaffolds have been described. In this work, in order to create mutants of triterpenoid synthases able to produce diverse chemical scaffolds, a random approach was preferred to direct mutagenesis methods. In fact, little information is available on enzyme domains or single amino acids responsible for radical changes in the catalytic mechanism of the triterpenoid synthases. Thus, the construction of chimeric gene libraries, through swapping domain methods, could provide a large source of new chimeric molecules that allows for selection of interesting and not predictable enzyme variants.

All cDNA molecules used in this work were synthesized with an optimized codon usage for efficient expression in yeast *Saccharomyces cerevisiae*.

The application of DNA shuffling methods requires a certain homology between the DNA molecules involved in order to allow for an efficient recombination of the fragments during the reassembling process. For this reason the molecules have been organized into three groups of four cDNA sequences according to their similarity values. β -amyrin synthases and Cycloartenol synthases were organized in two different groups according to their original enzyme family. A third mixed group was assembled with cDNAs of two β -amyrin and two lupeol synthases, different enzymes that catalytically act on the 2,3 epoxy-squalene with similar mechanisms. The DNA sequences in each pre-assembled group of molecules shared high nucleotide similarity, from ~73% to ~86%. (**Fig. 4.1**).

a. Beta-amyrin synthase and Lupeol synthase cDNAs

LUS (EV42)	100%			
LUS (EV43)	73,03%	100%		
bAS (EV48)	76,01%	79,09%	100%	
bAS (EV49)	75,53%	78,14%	80,85%	100%
	LUS (EV42)	LUS (EV43)	bAS (EV48)	bAS (EV49)

b. Beta-amyrin synthase cDNAs

bAS (EV47)	100%			
bAS (EV48)	86,46%	100%		
bAS (EV49)	80,39%	80,85%	100%	
bAS (EV50)	79,13%	80,58%	82,18%	100%
	bAS (EV47)	bAS (EV48)	bAS (EV49)	bAS (EV50)

c. Cycloartenol synthase cDNAs

CAS (EV52)	100%			
CAS (EV53)	85,2%	100%		
CAS (EV54)	82,34%	81,57%	100%	
CAS (C405)	80,93%	81,46%	78,33%	100%
	CAS (EV52)	CAS (EV53)	CAS (EV54)	CAS (C405)

Figure 4.1. Nucleotide similarity values between the sequences of triterpenoid cyclase cDNAs organized in three different groups for the application of DNA shuffling methods.

In order to evaluate the best experimental conditions for the construction of chimeric DNA libraries different protein engineering methods were investigated.

On the basis of the original DNA family shuffling protocol (Cramer et al., 1998) an equimolar mixture of cDNAs, PCR amplified, was subjected to DNaseI restriction reaction. The mixture of randomly generated fragments was further purified and reassembled in a primerless PCR step, in which no other DNA content was added. During the reassembling PCR, through reiterative steps of denaturation, annealing and elongation, the fragments primed to each other based on their homology and recombined to form full length molecules (**Fig. 4.2**).

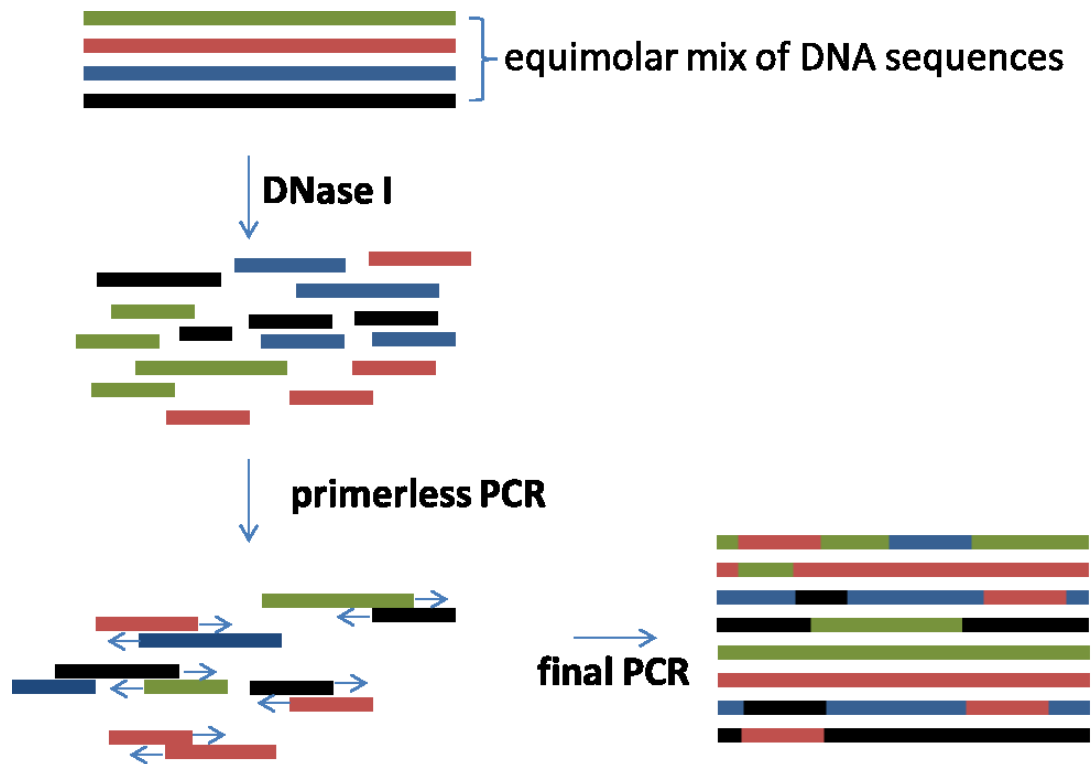


Figure 4.2. Graphic representation of the steps involved in a typical DNA family shuffling process. The parental molecules, indicated by different colours, are organized in equimolar mixture and randomly digested by DNaseI. DNA fragments are collected and used in a reassembly (primerless) PCR. Final chimeric molecules are PCR amplified from the mixture by adding universal external primer.

The successful application of the DNA family shuffling method required a precise control of the DNaseI digestion reaction to obtain fragments of desired length and an efficient reassembling process during the primerless PCR. The favourable outcome of these evolutionary strategies depends on the efficient recombination of heterologous fragments during the primerless PCR based on reiterative denaturation and annealing steps.

In this case the protocol was successfully adapted in order to obtain amplification of full length final molecules that indicated correct conditions for the reconstruction of the sequences. Nevertheless, this aspect did not confirm that conditions were optimal for heterologous recombination during the primerless PCR. In fact, sequences analysis revealed low recombination rates (1 to 6 recombination events) per molecule and high presence (at least ~25%) of parental non-modified molecules in the mixture. Parental molecules were found at higher percentages especially in the mixed group of lupeol and β -amyrin synthase cDNAs (**Fig. 4.1a**) where a lower presence of high homology regions, favourable for recombination between the molecules, promoted the reassembly of wild-type sequences during the primerless PCR.

Strong presence of point mutations with a rate of ~0.5% were detected in the final mix of molecules and often caused the appearance of stop codons and, therefore, translation of truncated non-functional enzymes. In fact, the enzymatic DNA fragmentation used in this method most likely contributed to create very small DNA fragments that could prime to homologous regions causing single nucleotide mismatches during amplification. In addition a laborious control of the DNaseI reaction due to the relatively long nature of the starting cDNAs (2.3 Kb) did not guarantee easy handling and reproducibility of this method.

All these reasons and the absence of a direct high-throughput screening system to easily isolate the recombinant functional DNA molecules in millions of new products led to the investigation of new strategies for the construction of chimeric DNA libraries with minimal contamination of unmodified parental sequences and high control of the recombination process.

A new method inspired by the “degenerate oligonucleotide gene shuffling” (Gibbs et al., 2001) was finally conceived and properly adapted for the purposes of this work.

This method is a semi-synthetic DNA family shuffling process with few but relevant differences with respect to the DNA family shuffling protocol previously employed. The use of DNaseI is avoided and substituted with PCR amplification of short overlapping fragments from the starting sequences. The regions selected for primers design correspond to the regions of reassembly of the DNA fragments during the primerless PCR (**Fig. 4.3**). Therefore, in this case, recombination is guided by the operator and strictly based on sequence homology. This semi-synthetic shuffling method does not allow for a random fragmentation but provides flexibility and full control of the number and position of possible crossover events in the chimeric final products. Moreover, it gives the ability to drive the shuffling of part of the genes and single DNA domains for more precise domain-function investigations (**Fig. 4.3**).

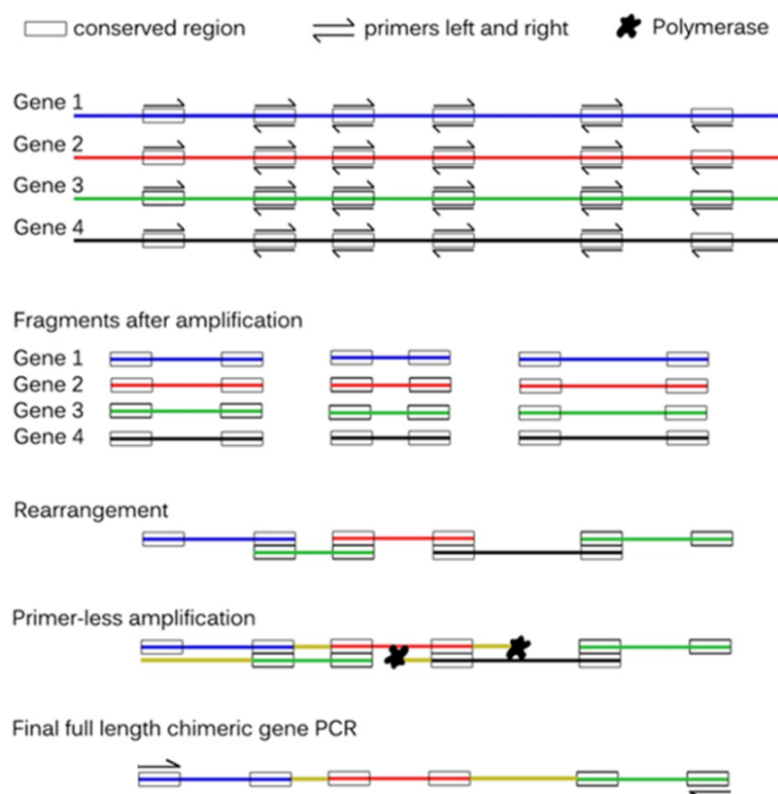


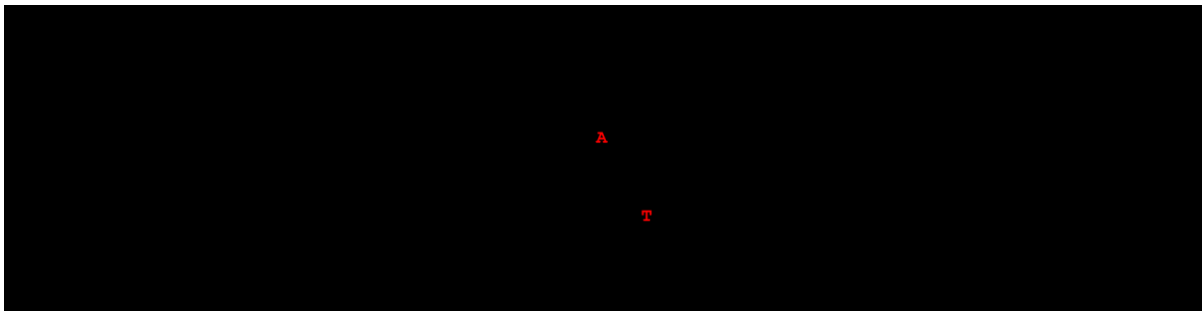
Figure 4.3. Schematic representation of the DNA semi-synthetic shuffling protocol. Different colours indicate different parental molecules processed together. Overlapping DNA fragments are amplified from all the starting sequences. Equimolar mixture of the fragments are set and subjected to recombination PCRs where the fragments can overlap and recombine until the full length molecules are created. Final amplification with external primers ensures the preparation of the new sequences for cloning and further analyses (Ritler D. bachelor thesis).

First, for each group of triterpenoid synthase cDNAs to be processed, the DNA regions with highest similarity have been identified. Based on these regions, short overlapping common primers (10-15 bp) of opposite orientation were designed and used for the PCR amplification of DNA fragments. The primers bound efficiently to the DNA of all members of the same group in each conserved region selected (**Fig. 4.4a**). Ten couples of common primers plus two universal primers designed upstream and downstream of the sequences (within the recipient vector) were used for the amplification of eleven DNA “bricks” (100 -500 bp) from each member of the group (**Fig. 4.4b**).

Overlapping primers with opposite orientation, designed on the same conserved regions, represented the extreme overlapping ends of two adjacent amplified DNA fragments and were responsible for subsequent recombination processes in the primerless PCR (**Fig. 4.4b**). In fact, the use of a common pair of primers for the amplification of each fragment from all the different starting

molecules ensured that the overlapping regions between two adjacent fragments were identical, whether these were amplified from the same sequence (homo) or from different sequences (hetero) of the group. Therefore, as a consequence, formation of hetero-duplex and homo-duplex recombination events during primerless PCR had the same probability to occur. This feature was specifically conceived to enhance the number of recombination events in the final mix of molecules.

a.



b.

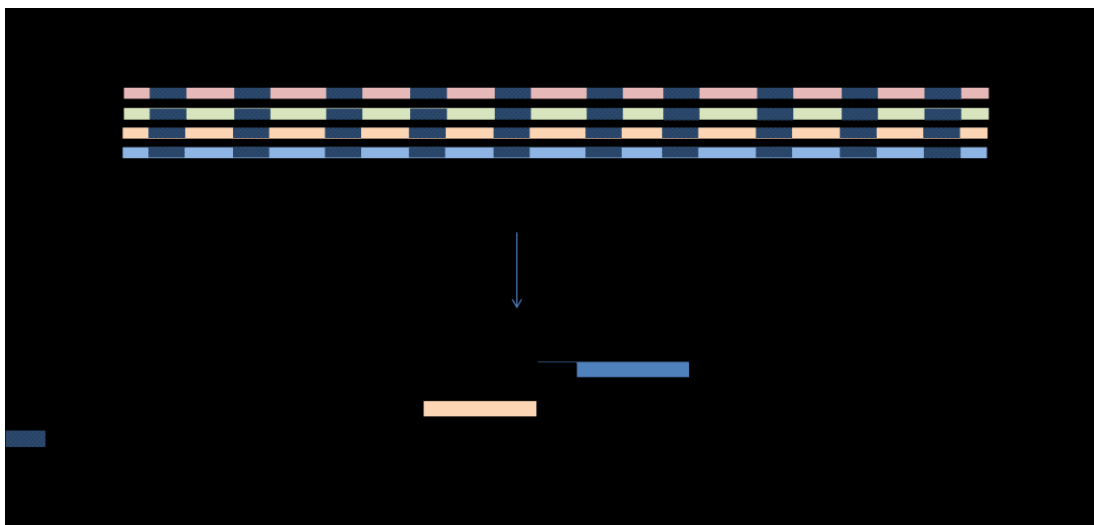


Figure 4.4. **a)** Example of primers designed for the semi-synthetic DNA shuffling. The cDNA sequences are aligned and one conserved regions is shown (bold). Short primers able to bind efficiently all the four templates were generated. Red nucleotides indicate mismatches in the sequences alignment. **b)** Schematic representation of cDNAs in the original recipient plasmid. Arrows indicate the primers used for fragments amplification. Two fragments are represented to emphasize the ending common regions of the DNA "bricks" that allow recombination during the primerless PCR. Different colours indicate different cDNAs treated together.

All DNA fragments generated from the same group of cDNA molecules were organized in equimolar mixtures and subjected to primerless PCR reactions. Upon several cycles of denaturation, annealing

and elongation steps the fragments bound to each other and gradually reassembled to higher molecular weight DNA molecules. As a result, from the first 50 cycles of primerless PCR, a clear DNA smear was typically detected through DNA electrophoresis and ethidium bromide staining (**Fig. 4.5, PCR 1**). Generally, if the DNA mixture did not reach the required length of 2,3 Kb, 25 additional cycles of amplification were performed before progressing to the final amplification (**Fig. 4.5, PCR 2**).

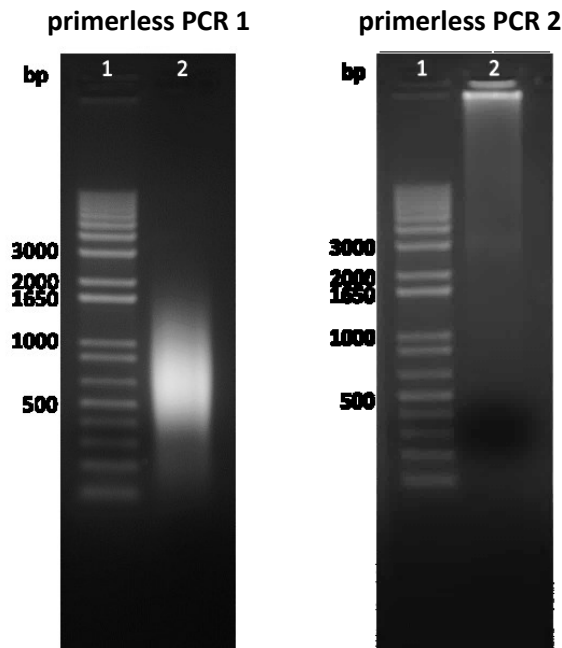


Figure 4.5. Agarose gel electrophoresis of typical primerless PCR products. Lane 1: 1 kb Invitrogen molecular DNA ladder. Lane 2: amplification products after the primerless PCR 1 and 2, respectively. In both cases a clear DNA smear is visible. An increase in molecular weight can be noticed through comparison between the products of the two subsequent PCRs.

The high molecular weight DNA mixture detected on agarose gel revealed a successful reassembling process and was then used, at different dilutions, as template for the amplification of the final sequences. Couples of external primers carrying target restriction sites for subsequent cloning steps have been employed for the amplification. As a result of the final PCR, a DNA product corresponding to 2.3 kb was typically detected through DNA electrophoresis and purified from agarose gel (**Fig. 4.6**). Successful amplification of the final full length molecules strictly depended on the quality of the templates employed. A specific and clean DNA band of the right size usually appeared when high dilutions of the primerless PCR products were employed as templates, reducing the presence of DNA not properly assembled in the reaction mixtures.

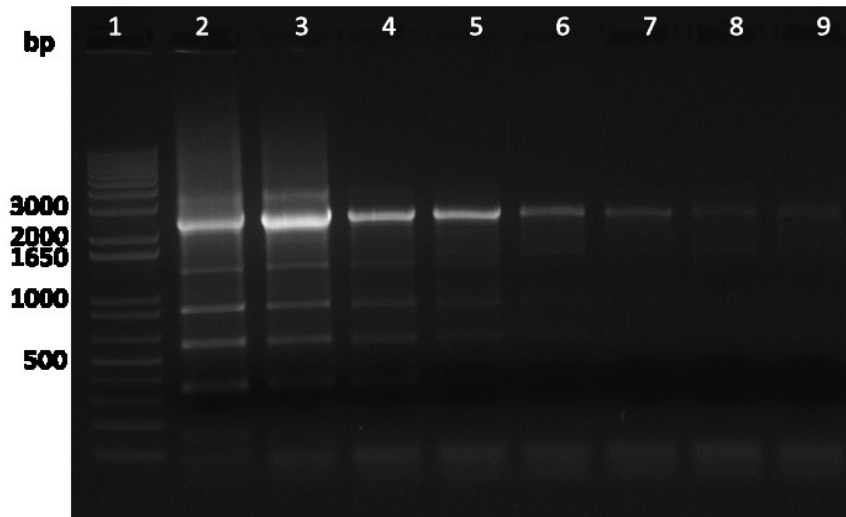


Figure 4.6. Agarose gel electrophoresis of a typical final PCR. Each lane (2-9) represents the product of a single PCR reaction performed on a template at increasing dilutions of 1, 1/3, 1/10, 1/30, 1/100, 1/300, 1/1000, 1/3000 respectively. Lane 1: 1kb DNA ladder. Specific bands are of 2.3 Kb. Unspecific products are visible in the first lanes (2 to 6) of the agarose gel .

After gel purification the mixture of sequences was digested with appropriate restriction enzymes and cloned into a group of recipient DNA entry vectors. Entry vectors are special DNA plasmids carrying all the basic elements for selection and plasmid amplification in *E.coli* (e.g. antibiotic resistance, *ORI*) together with combinations of yeast promoters and terminators (**Fig. 4.7**).

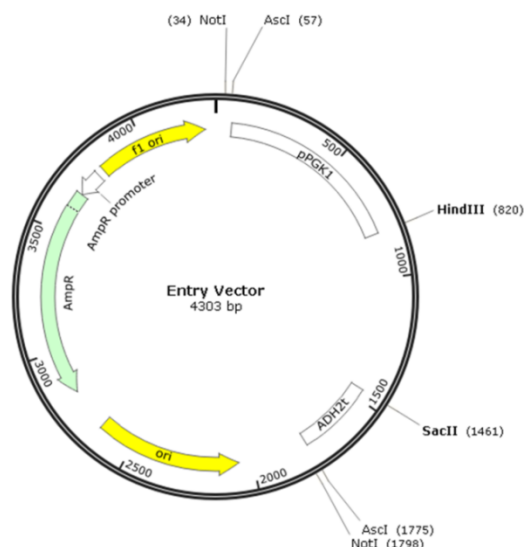


Figure 4.7. Plasmid map of a typical entry vector. The DNA molecules obtained through DNA semi-synthetic shuffling were cloned between the restriction sites of *HindIII* and *SacII*. Different combination of yeast transcription promoters and terminators are present in the different vectors used as recipients. All the promoter and terminator combinations of the vectors employed are listed in **Table 7.3**.

After cloning, in order to verify the success of the recombination process in the applied semi-synthetic shuffling, 60 DNA products were isolated and sequenced.

Sequence analyses revealed the positive outcome of the method (**Fig 4.8**). In fact, high numbers of recombination events (from 6 to 11) between heterologous DNA “bricks” were detected in each of the sequences analysed. Point mutations were significantly reduced (0.08%) due to the absence of DNaseI fragmentation step and to the use of proof reading high fidelity DNA polymerases in the reassembling process (Zhao and Arnold, 1997). Moreover, fragments from all parental cDNAs were represented in the new sequences analysed but contamination with full-length parental sequences was never detected.

From the analysis of the sequences it was noticed that the distribution of the single DNA fragments in the chimeric final molecules was not always uniform. In fact, some of the DNA “bricks” seemed to occur more frequently than others (**Fig. 4.8**). This discrepancy in the results with respect to the theoretical previsions, was most likely due to slight differences in the amount of fragments during the setting of equimolar mixtures for the primerless PCR or to a higher quality of some amplified DNA “bricks” that were then preferred in the reassembling process.

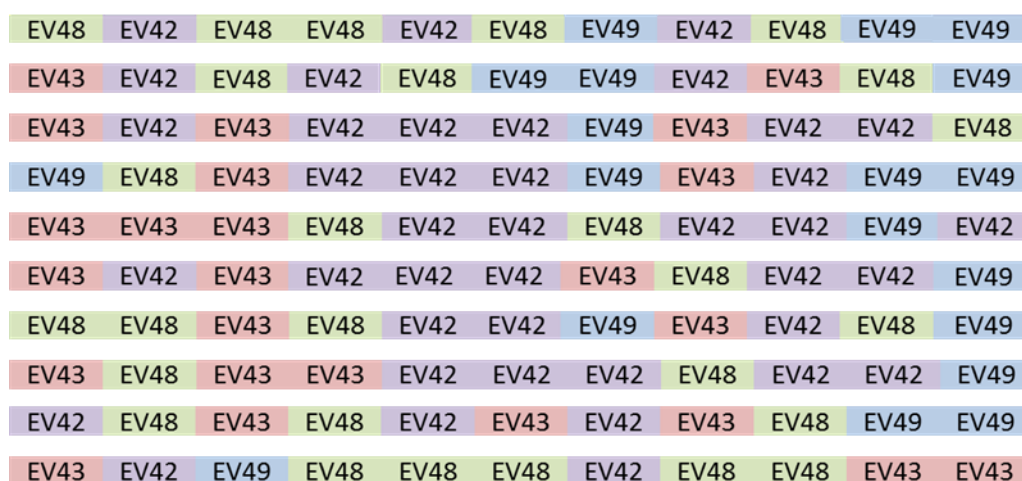


Figure 4.8. Schematic representation of 10 real DNA sequences (5'-3') obtained through the application of semi-synthetic DNA shuffling to families of triterpenoid synthases cDNAs. Every colour indicates a different origin of the fragment. EV42, EV43 and EV48, EV49 are the names for two lupeol synthase and two β -amyrin synthase cDNAs, respectively (**Table 4.1**).

Comparison between the recombination regions of chimeric DNA molecules obtained from the application of both the random and not random DNA shuffling methods previously described, revealed that in almost all the chimera analysed, recombination occurred at the same regions of the

sequences, characterized by high homology. This aspect relies on the fact that both methods are based on homologous recombination and therefore, regions selected for primers design in the semi-synthetic shuffling method frequently corresponded to the regions of recombination of the sequences obtained through the original DNA family shuffling method. This last aspect added more value to the DNA semi-synthetic shuffling method developed in this work, since it efficiently mimics the random method but avoiding at the same time the formation of wild type sequences in the final mixture.

Finally, the semi-synthetic method resulted in a highly reproducible, adaptable and efficient protocol for the construction of chimeric DNA libraries. Although it does not use random fragmentation due to the preselection of the fragments to be recombined, it always leads to the presence of high numbers of recombination events in the final sequences.

The number of predicted products for each group of cDNAs, through application of DNA semi-synthetic shuffling was 4^{11} (11 fragments and 4 genes per group). The DNA semi-synthetic shuffling protocol developed was applied several times to the three groups of triterpenoid synthase cDNAs to ensure the highest representation of different chimeric molecules in the final mixture prior to proceed with subsequent experimental steps. Based on recombination, cloning and transformation efficiency, the size of the chimeric cDNA library into different entry vectors was roughly estimated to be between 8 and 9 million of sequences.

eYACs construction

*In this chapter the investigation of a multi-cloning system based on expressible yeast artificial chromosome (eYACs) is described. This technology, developed at Evolva SA, allows for the simultaneous expression of a large number of genes in the yeast *Saccharomyces cerevisiae* and represents a powerful method for the reconstruction of complex biosynthetic pathways in this host organism. Here, the eYACs were assembled with expression cassettes of cDNA molecules essential to reconstitute the biosynthetic pathway of plant triterpenoids.*

In order to simultaneously express high numbers of chimeric and parental triterpenoid synthase cDNAs in yeast, expressible yeast artificial chromosomes (eYACs) were used. eYACs are powerful tools for multiple cloning and expression of heterologous genes in yeast and for reconstruction of exogenous or new biosynthetic pathways.

In this work eYACs were aimed to be employed for the coordinate and controllable expression of high numbers of triterpenoid synthase enzymes that are potentially able to enzymatically act on 2,3 epoxy-squalene converting it into different triterpenoid backbones. Part of the cDNA library previously prepared was therefore used for construction of eYACs. The mixture of entry vectors carrying chimeric and parental cDNAs has been purified from *E.coli* and processed with *NotI* and *Ascl* restriction enzymes. After digestion, the two main fragments, expression cassette and vector backbone respectively, were released together with two “stopper” DNA fragments (**Fig. 4.9**). Restriction with *Ascl* and *NotI* liberated the expression cassettes and the vector backbones with incompatible sticky ends. Before proceeding with the expression cassettes concatenation and in order to ensure a successful ligation of the cassettes, the “stopper” fragments were sequestered away by adding short competitive oligonucleotides to the mixture (**Fig. 4.9**). Through denaturation and fast cooling steps, the competitive oligonucleotides added formed a dsDNA with the complementary strand of the stopper fragments, preventing their renaturation. This process allowed for the formation of new double short blunt ends DNA strands that could not participate to the concatenation process (**Fig. 4.9**). After this step the DNA mixture consisted of vector backbones with *NotI* sticky ends, expression cassettes with *Ascl* sticky ends, and short dsDNA fragments with blunt ends.

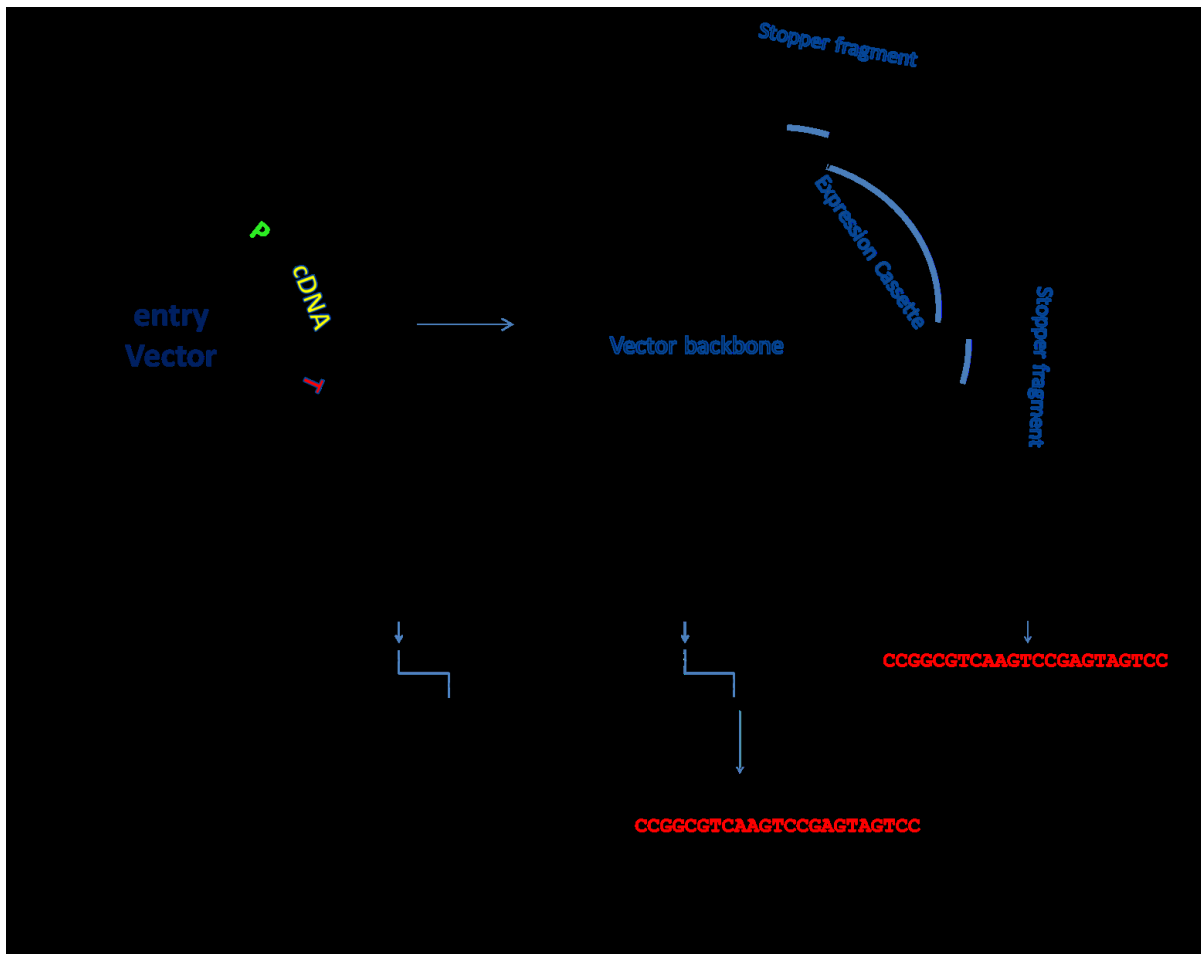


Figure 4.9. Steps involved in the preparation of the expression cassettes for the concatenation reaction. The short stopper fragments if kept in the mixture could impair the concatenation process. Synthetic competitive oligonucleotides are ssDNA able to efficiently bind one strand of the stopper fragments and create dsDNA with blunt ends that cannot participate to the concatenation process.

The expression cassettes were randomly concatenated by a standard ligation process into long chains of high molecular weight DNA (**Fig. 4.10b**). Subsequently chromosome arms carrying yeast elements for stability, duplication and segregation of the chromosomes were added to the ends of the DNA concatamers to create linear eYAC molecules (**Fig. 4.10c**). The mixture was then transformed into yeast spheroplasts of strain EYS1019 (genotype in Table 8.1). The presence of two different auxotrophic markers in the telomeres and in the internal part of the chromosomes, respectively, allowed an efficient selection of colonies that stably maintained the artificial chromosomes after several generations (**Fig. 4.10d**).

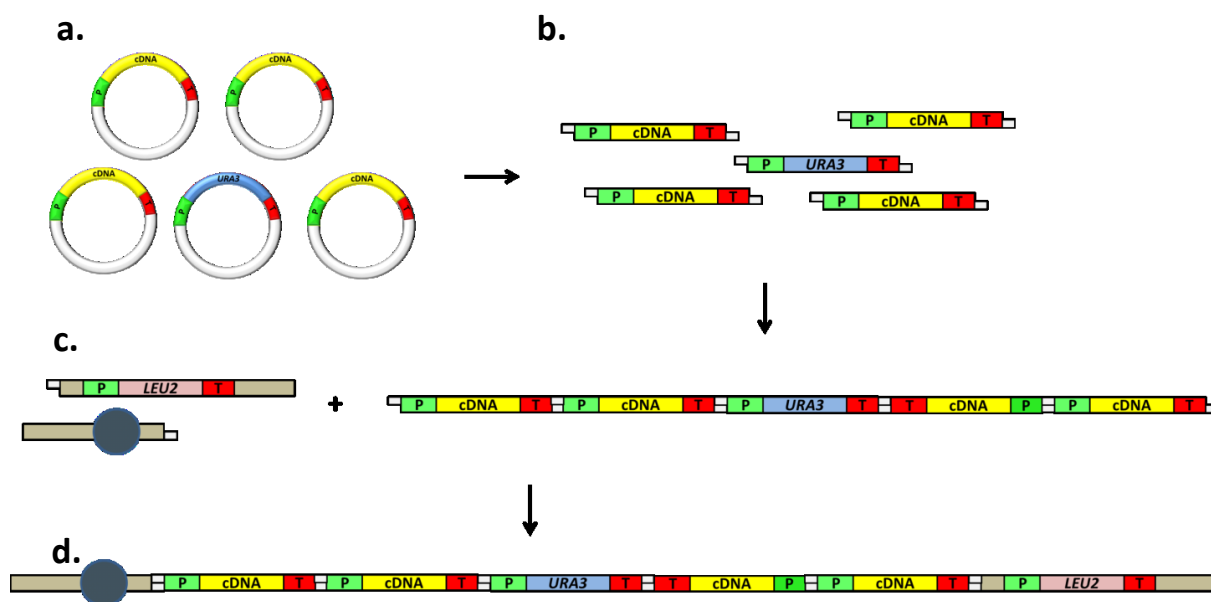


Figure 4.10. Schematic representation of eYACs assembly. **a)** Expression cassettes of cDNAs and genes of interest in the recipient entry vectors. **b)** Digestion process releases all the expression cassettes with compatible sticky ends (process described more in detail in figure 4.9). **c)** Random concatenation of the expression cassettes in high molecular weight DNA and subsequent chromosome arms (brown) addition to the ligation mixture. The arms carry all the elements for the functionality and stability of the chromosomes; centromere (circle), telomeres, and the autonomous replicating sequence (not shown). In addition, they carry an expression cassette for auxotrophic selection marker (*LEU2*). **d)** Arms are ligated to the ends of the cassettes concatamers.

According to the original protocol, the expected eYACs average size was 130 Kb corresponding to approximately 45 expression cassettes (~2.9 Kb) per chromosome (Naesby et al., 2009).

During eYACs preparation many aspects of the method have been carefully evaluated in order to ensure the best results. For example, self-recombination events due to high similarity of the sequences used in the construction strongly influenced the favourable outcome of the experiments. In fact, preparations performed exclusively using libraries of chimeric and parental triterpenoid synthase cDNAs did not produce any functional eYAC and consequently no colonies were detected on selection plates after transformation. To overcome this technical problem, additional eYACs preparations were performed trying to dilute the presence of high similarity sequences in the mixture by adding in different amounts of foreign DNA material, not relevant for expression in yeast and appropriately prepared to be concatenated as connectors between the main expression cassettes. The addition of external DNA material during eYACs assembly decreased the number of chimeric and parental triterpenoid synthase expression cassettes per chromosome since the final average size of the chromosomes was not expected to change. This procedure led to a successful

concatenation process and created more stable eYACs with respect to the previous trials. With the optimized protocol few hundreds of yeast colonies containing randomly assembled eYACs resulted from each transformation. In order to estimate the quality and content of the chromosomes, approximately 20 positive colonies were selected and grown in liquid medium. The organic phases were extracted and finally analysed through GC-MS for detection of triterpenoid molecules. Despite the ability of the cells to grow on double selection media suggested for the presence of eYACs, no detection of known triterpenoids was revealed through GC-MS in any of the samples analysed. The negative outcome was addressed, with high probability, to the short nature of the chromosomes due to residual self-recombination processes and to the presence of the non-functional DNA material that provided more stability to the final concatamers but at the same time significantly reduced the number of functional expression cassettes represented per chromosome.

In order to investigate whether the absence of triterpenoids production in the yeast harbouring the eYACs could be also due to the impaired functionality of the triterpenoid synthases (natural and chimeric), four parental β -amyrin synthases cDNAs (Accession Numbers: AB037203, AB009030, AF478453, AB014057) and twenty chimeric sequences were individually sub-cloned and expressed in yeast strain EYS1019. After cultivation in inducible media in order to trigger the expression of the cDNAs cloned downstream the *GAL1* promoter, 5 ml of each culture were processed through an ethanol/hexane extraction procedure and the organic phases were analysed by GC-MS.

Saccharomyces cerevisiae wild type cells contain ergosterol, synthesized from 2,3 epoxy-squalene via lanosterol, as a major sterol component but do not produce structures similar to the β -amyrin. For this reason plant triterpenoids can be easily detected in GC profiles of yeast extract.

Yeast cells that heterologously expressed β -amyrin synthases of *Glycyrrhiza glabra* (AB037203) and *Panax ginseng* (AB009030) accumulated a detectable compound with a mass spectral fragmentation pattern identical to the standard β -amyrin (**Fig. 4.11**). The areas of the two β -amyrin peaks detected indicated a strong difference in the activity of the two wild type synthases when expressed in yeast.

The cells expressing β -amyrin synthases of *Medicago truncatula* (AF478453) and *Panax ginseng* (AB014057) did not produce any detectable known triterpenoid despite the fact that their functionality in yeast was already demonstrated in the past (Kushiro et al., 1998; Iturbe-Ormaetxe et al., 2003).

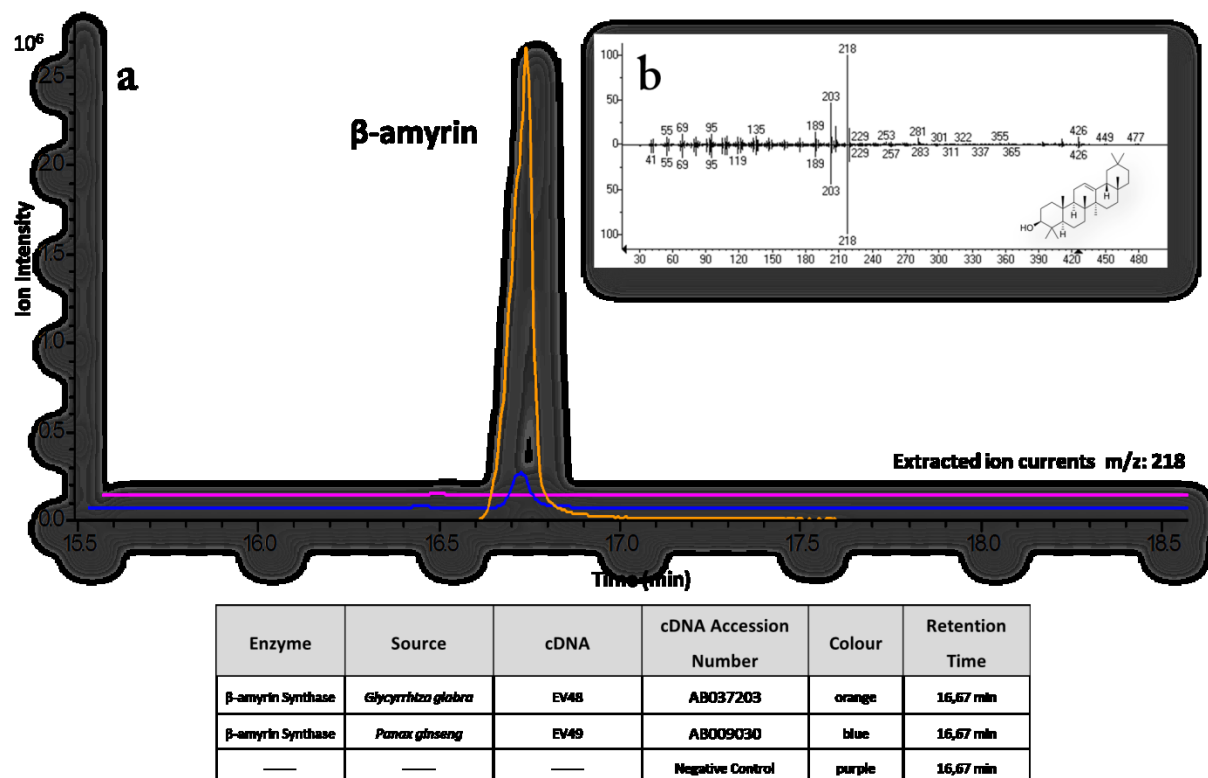


Figure 4.11. a) GC-MS profiles of EYS1019 yeast strain expressing β -amyrin synthases from *Glycyrrhiza glabra* (orange) and *Panax ginseng* (blue). The peak corresponding to β -amyrin was detected at 16.67 minutes of retention time. As negative control the strain was transformed with the empty plasmid and after cultivation in galactose selection medium the organic extract was analysed (purple). **b)** Mass spectrum of the detected peak (upper panel) compared with the mass spectrum of standard β -amyrin (lower panel).

The GC-MS analyses of yeast cells expressing wild type synthases were followed by the analyses of yeast expressing for chimeric sequences obtained through the DNA semi-synthetic shuffling. GC-MS confirmed that three of the chimeric enzymes tested produced β -amyrin with different efficiencies respect to the wild type parental enzymes (**Fig. 4.12**). These results strongly suggested that the DNA mutagenesis method developed in this work leads to functional enzymes with modified catalytic efficiencies and confirmed the good quality of the cDNA library previously constructed.

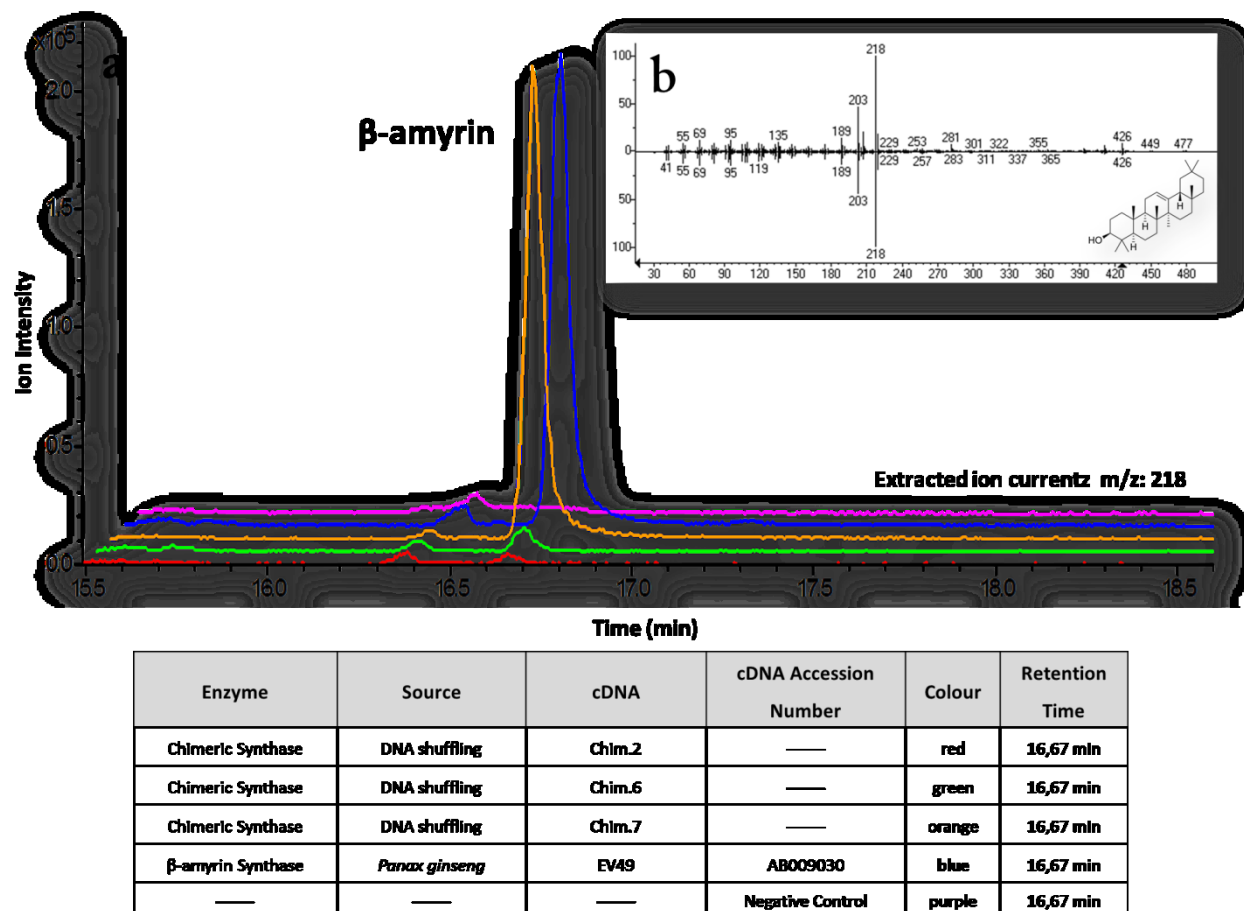


Figure 4.12. a) GC-MS profiles of EYS1019 yeast strain expressing chimeric enzymes, obtained through DNA semi-synthetic shuffling (red, green, orange), and the β -amyrin synthase from *Panax ginseng* (blue). A peak corresponding to β -amyrin was detected at 16.67 minutes of retention time. As negative control, the strain was transformed with the empty plasmid and the extract analysed after cultivation in galactose (purple). b) Mass spectrum of one of the detected peaks (upper panel) compared with the mass spectrum of standard β -amyrin (lower panel).

Different catalytic features such as product specificity, associated with the new chimeric enzymes expressed in yeast, were neither demonstrated nor excluded. In fact, the GC-MS method applied, although was optimal for detection of triterpenoid backbones, did not guarantee a wide screening of chemical scaffolds. Therefore eventual new products, due to the reactions catalysed by the chimeric enzymes, with different structures with respect to the triterpenoid could not be detected. For this last purpose much more advanced analytical analyses and strategies would be needed.

Yeast-based 11 β -HSD1 assays

Finalization of this innovative strategy for intracellular production and screening of 11 β -HSD1 inhibitors involved the development of cellular systems where the functionality of the target enzyme is connected with an output easy to be detected. Yeast cells in the synthetic biology platform here investigated are meant to be first, generator of compounds and second, screening systems for the activities of these compounds against the target enzyme (Klein et al. 2014). For this purpose this chapter describes the construction of two yeast strains, RVY97 and RVY102, for screening of 11 β -HSD1 inhibitors.

Yeast has emerged in the last decades as a powerful organism for the study of many human target enzymes. The deep genetic information available on this system has allowed it to become an increasingly popular model for pharmacological and drug discovery studies that can be used to analyse the effects of drugs *in vivo* during initial stages, before tests are performed in mammalian systems. Compared to higher eukaryotes, yeast cells are more economical, easier to grow and can be genetically manipulated with greater ease. Hence, the availability of well-established genomic methods and resources makes the budding yeast an extremely valuable asset for the screening of molecules with possible pharmaceutical applications (Barberis et al., 2005). Moreover, coupled with technologies that allow the construction of complex biosynthetic pathways yeast cells represent important tools for simultaneous synthesis and screening of heterogeneous compounds with therapeutic features.

Construction and validation of the assay strain RVY97

In this study the assay strain RVY97 was conceived in order to have a direct connection between the activity of 11 β -HSD1 and a fluorescent output. The human 11 β -HSD1 enzyme, expressed in yeast cells, reduces the cortisone provided in the growth medium, thereby converting it to cortisol, the active form of the steroid hormone. Cortisol binds to the glucocorticoid receptor (GR), also heterologously expressed in yeast. The active complex translocates into the nucleus, promoting the expression of γ EGFP via specific interaction with the glucocorticoid response element (GRE) upstream of the reporter gene. In this strain inhibition of 11 β -HSD1 causes a decrease of the green fluorescence (**Fig. 4.13**).

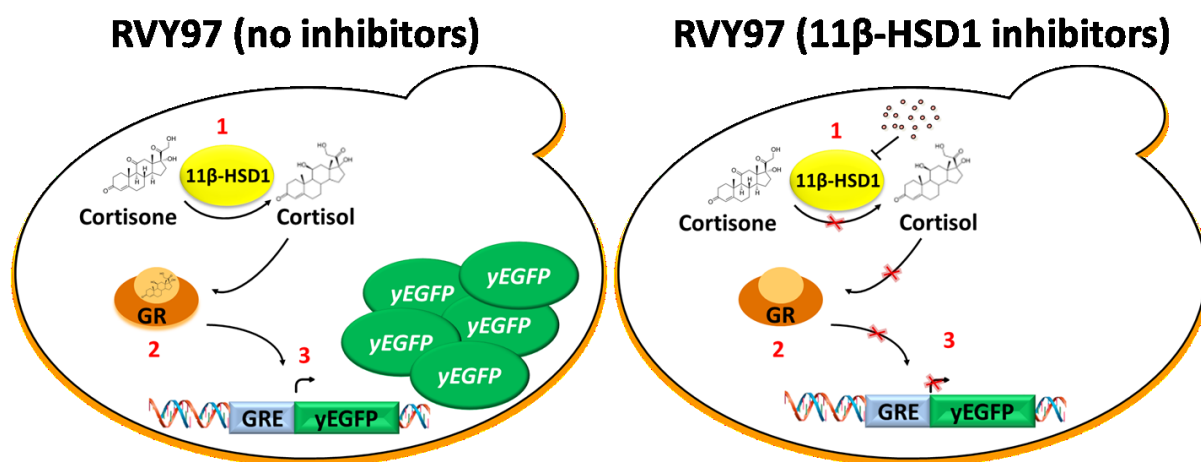


Figure 4.13. Schematic representation of the screening strain RVY97. In strain RVY97 the cortisone provided in the growth medium is converted to cortisol inside the cell by the enzyme 11β-HSD1. Cortisol binds the GR inducing assembly of an active complex that translocates to the nucleus. Through binding of the complex to the GRE, expression of the yEGFP reporter protein is triggered. In the presence of 11β-HSD1 inhibitors, cortisol is not produced, the GR is inactive and yEGFP not expressed.

The yEGFP reporter gene was placed under the control of 3xGRE+CYC1-TATA promoter that upon binding of the murine glucocorticoid receptor efficiently activates a downstream reporter constructs in yeast (Picard et al., 1990). The promoter 3xGRE+CYC1-TATA included the insertion at position -178 of the truncated yeast CYC1 promoter of three tandem 26-bp oligonucleotides derived from the murine tyrosine aminotransferase GRE (Garabedian and Yamamoto, 1992). The construct was integrated as a single copy into the ECM3 locus of chromosome XV of the S1502B yeast strain genome. The mouse cDNA *Nr3c1* codifying for the glucocorticoid receptor (GR) was cloned into a 2μ vector under the control of the GAL1 promoter and transformed in the S150-2B strain containing the integrated GRE-yEGFP construct. The resulting yeast strain was named as RVY96.

To assess the functionality of the fluorescent reporter construct, γ EGFP expression experiments were performed with strain RVY96 in presence of glucocorticoids.

Cortisol or dexamethasone, able to bind efficiently to the glucocorticoid receptor, were added to yeast cultures and the expression of γ EGFP within the RVY96 yeast population was monitored through fluorescence microscopy. The cooperation between the GR and the *GRE- γ EGFP* in the RVY96 strain was confirmed. Dexamethasone treatments significantly triggered the expression of γ EGFP in the population and on the contrary no fluorescence was detected in the control samples in which the cells were treated with DMSO (Fig. 4.14).

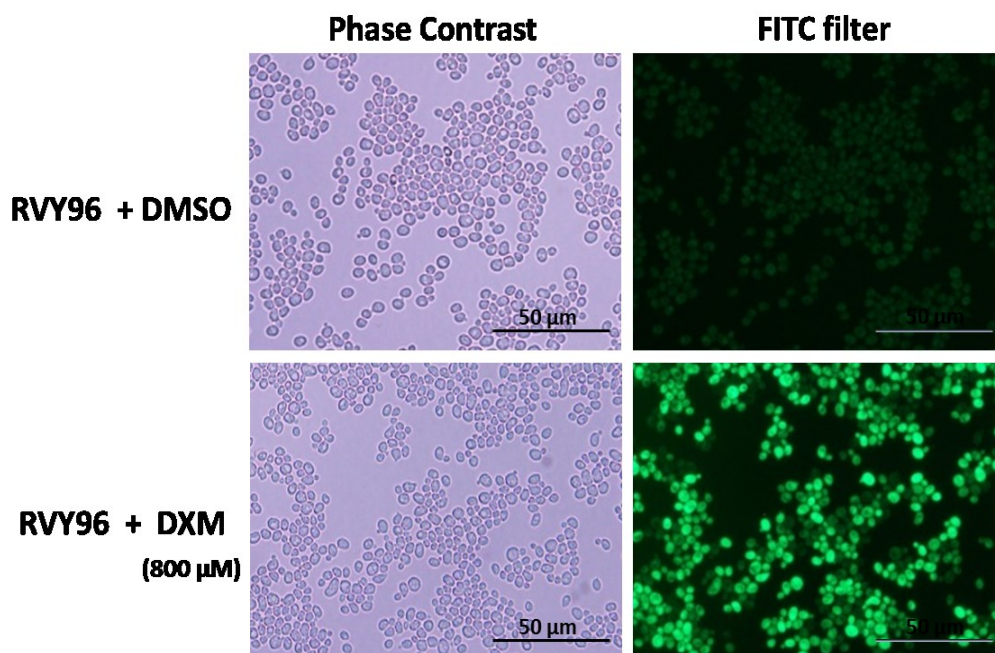


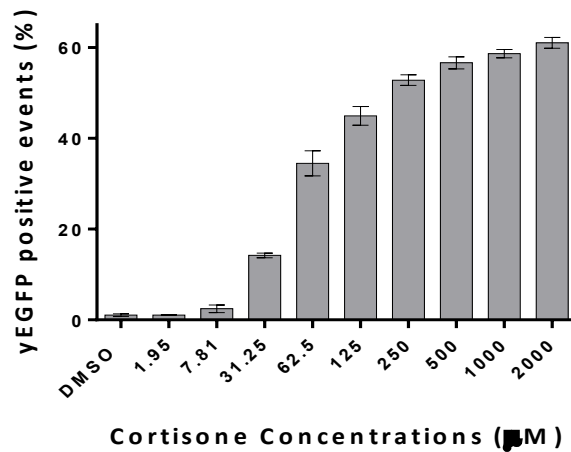
Figure 4.14. Effect of dexamethasone on γ EGFP expression in RVY96 yeast strain. DMSO (control) and 800 μ M dexamethasone (DXM) were added to the cell cultures. γ EGFP expression was detected in the population treated with dexamethasone and not in cells treated with DMSO. The analyses were carried out 6 hours after the addition of DMSO and dexamethasone, respectively.

In order to finalize the construction of the assay strain RVY97, the human cDNA *HSD11B1*, codifying for the enzyme 11 β -HSD1, was cloned under the control of the *GAL1* promoter into a 2 μ vector and transformed into the strain RVY96. *GAL1* allowed a fine and simultaneous expression of 11 β -HSD1 together with the mouse GR in the resultant RVY97 strain and guaranteed functional collaboration of the two mammalian proteins for the final transactivation of the fluorescent reporter. The expression of 11 β -HSD1 in the RVY97 was confirmed through western blot analyses and the activity of the

enzyme expressed in yeast was investigated both *in vitro* (after enzyme purification) and *in vivo* through HPLC analyses of organic extracts from yeast cultures treated with cortisone (Manday Viswanathan P. master thesis).

In order to investigate for the functionality of the strain RVY97, FACS analyses were used to assay the expression of γ EGFP as response to the treatment with different cortisone concentrations, ranging from 1.95 μ M to 2000 μ M (**Fig. 4.15a**). As expected, green fluorescence in the treated population increased, due to the action of 11 β -HSD1, with increasing concentrations of cortisone, whereas in the absence of hormone no fluorescent signal was detected. Concentration of cortisone as low as 31.5 μ M were effective, leading to appearance of an unambiguous fluorescent signal connected to the activity of 11 β -HSD1. However, higher concentrations of cortisone were more desirable due to the stronger induction of reporter protein expression. Expression of γ EGFP, driven by 11 β -HSD1-dependent conversion of cortisone to cortisol, rose significantly upon treatment with a range of cortisone concentrations between 60 and 500 μ M. At higher concentrations a plateau of fluorescence intensity was reached (**Fig. 4.15a**). Of all hormone concentrations tested, 250 μ M cortisone guaranteed the best compromise between intensity of the generated fluorescent signal, solubility and low toxicity of cortisone added to the yeast population. FACS analysis and fluorescence microscope images of RVY97 cells treated with DMSO (control) and 250 μ M cortisone are shown in figure **4.15, b** and **c**, respectively. These results suggest the dependency of the fluorescent signal on the activity of 11 β -HSD1 on cortisone. In this strain the identification of putative inhibitors of the target enzyme is based on the loss of fluorescence in the treated cells. The specificity of the signal upon treatment with cortisone was confirmed with a strain in which the plasmid carrying the 11 β -HSD1 expression cassette has been replaced by the equivalent empty plasmid. No response to cortisone was detected in the negative control strain.

a.



b.

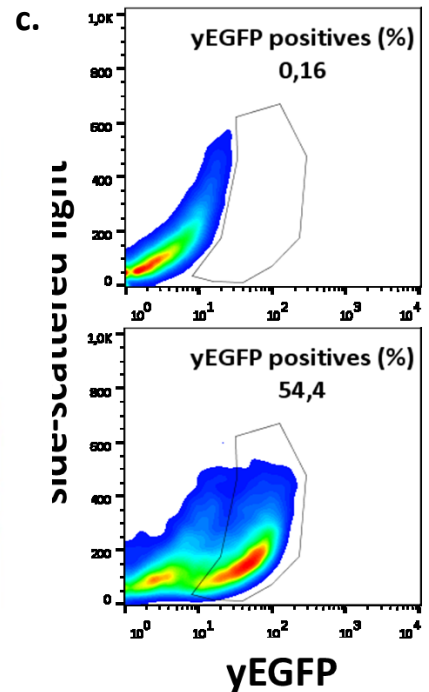
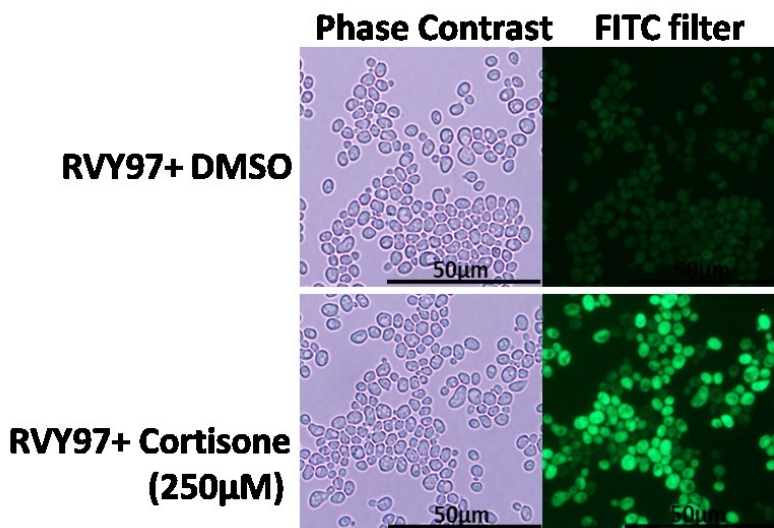


Figure 4.15. Effect of cortisone on yEGFP expression in yeast strain RVY97. **a)** Increased cortisone concentrations led to an increase of yEGFP expression in a concentration dependent manner. The treated RVY97 population (10^4 events) was analysed by FACS and the results are expressed in percentage of RVY97 cells showing yEGFP fluorescence. **b)** Fluorescence microscopy of strain RVY97 showed that cortisone treated cells displayed a strong fluorescent response to the hormone. **c)** FACS analysis of the strain RVY97 cultivated with DMSO (upper panel) and $250 \mu\text{M}$ cortisone (lower panel) enabled quantitative analysis of the hormone response of this specific strain. The gate highlights the RVY97 population positive for the yEGFP expression. All analyses were carried out 6 hours after the addition of DMSO and cortisone, respectively.

To address the validity of the assay for screening of putative 11 β -HSD1 inhibitors, a series of experiments with a synthetic inhibitor of this enzyme were performed. RVY97 strain was incubated with cortisone in presence of carbenoxolone (CBX), a synthetic hemisuccinyl ester derivative of glycyrrhetic acid. Carbenoxolone has been shown to exert potent but non-selective 11 β -HSD1 inhibitory activity and is frequently used as control in inhibition studies involving this target enzyme (Monder et al., 1989) Carbenoxolone behaves as a competitor of cortisone, binding to the active domain of 11 β -HSD1 (Castro et al., 2007; Su et al., 2008).

In order to validate the assay with this molecule, different inhibitor concentrations ranging from 0.1 to 3 μ M were added to 1 million RVY97 cells together with 250 μ M of cortisone. Increasing concentrations of carbenoxolone caused a gradual loss of γ EGFP expression, indicating a decreased activity of 11 β -HSD1 in the population with a complete absence of fluorescence at a concentration of 3 μ M of inhibitor, most probably associated with the complete inhibition of the enzyme (**Fig. 4.16**).

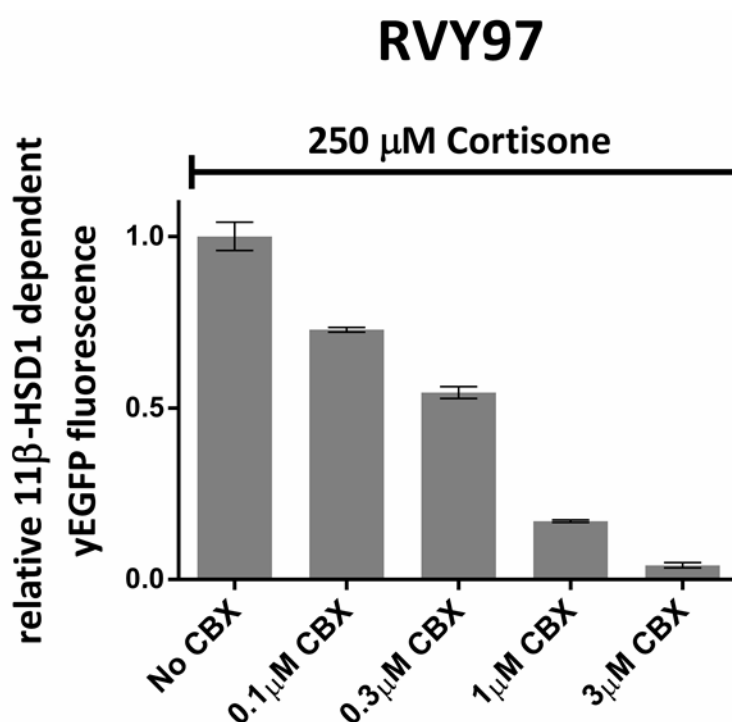


Figure 4.16. Effect of carbenoxolone (CBX) on 11 β -HSD1 dependent response in the strains RVY97. 11 β -HSD1 dependent γ EGFP fluorescence of strain RVY97 treated with 250 μ M cortisone and increasing CBX concentrations. Increasing concentration of carbenoxolone (up to 3 μ M) led to a decreasing green fluorescence in the yeast population indicating a progressive inhibition of 11 β -HSD1. The fluorescence of untreated cells (No CBX) was set at 1.

Construction and validation of the assay strain RVY102

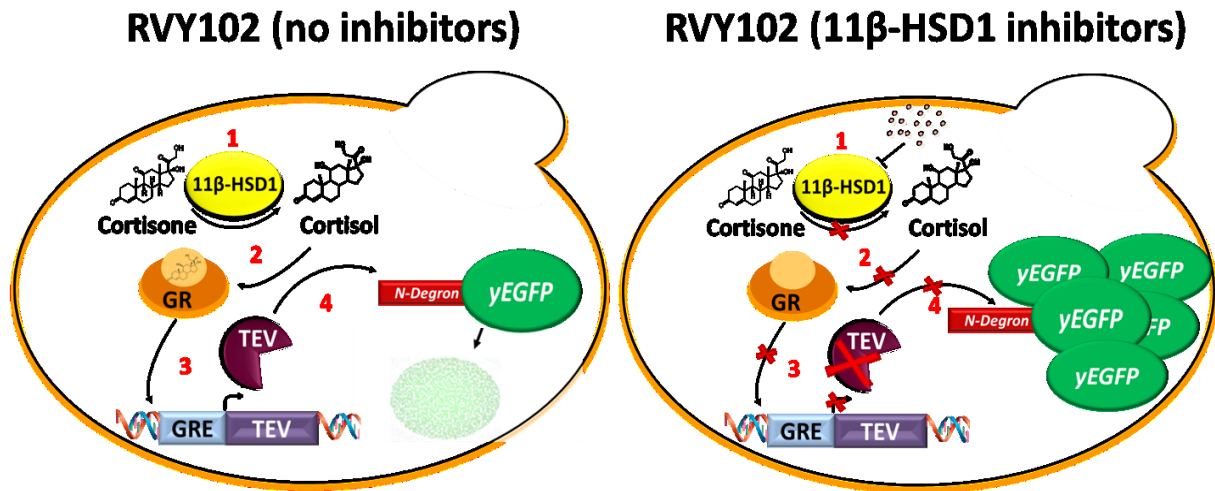
A robust cell-based screening assay relies on the strong correlation between an intracellular event and a univocal and easily detectable output. Screening systems with a positive readout guarantee the selection of hits from the analysis of large number of samples with minimum false positive results caused by unrelated intracellular events, such as impaired cellular viability or loss of an element of the assay system. To set up a more reliable assay for the screening of 11 β -HSD1 inhibitors a strain was constructed in which inhibition of 11 β -HSD1 is connected to a positive fluorescent readout. In contrast to strain RVY97 where the activity of the enzyme is directly connected with the expression of a fluorescent reporter protein, in strain RVY102 the reduction of cortisone, driven by 11 β -HSD1, is combined with a TEV protease induced protein instability (TIPI) mechanism (Taxis et al., 2009) (**Fig. 4.17a**). This mechanism reversely combines the activity of the target enzyme to the stability of a constitutively expressed reporter protein. The TIPI strategy exploits the presence of a TEV cleavage site in a reporter protein, in order to induce its degradation.

The reporter construct expresses a fluorescent fusion protein consisting of the red tandem dimer (td) Tomato, the TEV recognition site, an N-Degron destabilizing sequence, the SF3b155³⁸¹⁻⁴²⁴ protein domain and the γ EGFP (Taxis et al., 2009) (**Fig. 4.17b**).

As shown in figure 4.17a, the expression of the TEV protease under control of the glucocorticoid response element is driven by the active complex formed after specific binding of cortisol to the glucocorticoid receptor. Expression of the TEV protease, leads to cleavage of the reporter protein and to the exposure of a dormant N-Degron sequence. This sequence contains at its N-terminus the destabilizing amino acid phenylalanine followed by several lysine residues that, when exposed, serve as attachment sites for ubiquitin leading the protein to proteosomal degradation (**Fig. 4.17b**). The exposure of this destabilizing stretch of amino acids, after TEV protease cleavage, causes the C-terminal part of the reporter construct, including γ EGFP, to undergo degradation (Bachmair et al., 1986). The SF3b155³⁸¹⁻⁴²⁴ protein domain binds strongly to the protein p14, fused to TEV, thus enhancing processivity of the protease (Taxis et al. 2009). The tdTomato red fluorescent protein is used for normalization purposes, since it does not undergo degradation upon TEV protease activity. On the other hand, the γ EGFP reporter protein reversely monitors the TEV protease activity, as it is degraded upon TEV expression.

The described cascade in strain RVY102 directly couples the activity of 11 β -HSD1 to γ EGFP degradation. Inactivation of 11 β -HSD1 caused by inhibitors, leads to stability of the reporter protein and detection of an intact green fluorescent signal in the population.

a.



b.

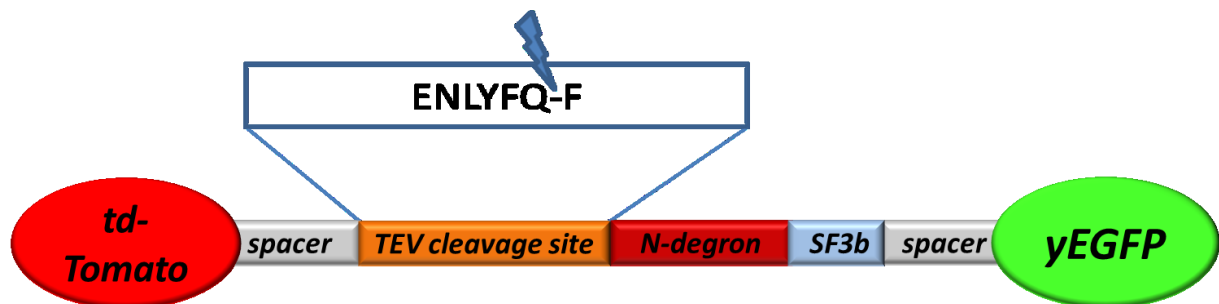


Figure 4.17. a) Schematic representation of the screening system RVY102. Cortisone added to the growth medium enters RVY102 cells and is reduced to cortisol by the enzyme 11β -HSD1. The active complex formed by binding of cortisol to GR triggers expression of the TEV protease which binds to its cleavage site and cuts the reporter fluorescent protein that then undergoes degradation. Presence of an 11β -HSD1 inhibitor will stabilize the reporter protein. b) Graphic representation of the reporter fusion protein expressed in strain RVY102. The fusion protein consists of the red tandem dimer (td) Tomato, the TEV recognition site, an N-Degron destabilizing sequence, the SF3b³⁸¹⁻⁴²⁴ protein domain and the yeast enhanced green fluorescent protein (yEGFP). Once expressed, the TEV protease cuts at its cleavage site on the reporter protein, leading to exposure of the N-Degron sequence and degradation of yEGFP. The TEV recognition sequence and the destabilizing amino acid (phenylalanine) are shown.

In order to construct the yeast strain RVY102, first, the *TEV* protease gene was cloned under the control of the promoter *3xGRE+CYC1-TATA* and the construct was integrated into the *ECM3* locus of chromosome XV of the S150-2B yeast genome. After, the N-Degron fluorescent reporter construct controlled by *GAL1* promoter was integrated at the *KIN1* locus of chromosome IV, and its expression confirmed through fluorescence microscopy of cells grown in galactose medium.

Furthermore, the *Nr3c1* expression construct, codifying for GR, was transformed and the resultant yeast strain, containing all the three elements, was named as RVY101. The functional cooperation between the GR, *GRE-TEV*, and N-Degron fluorescent protein was tested in RVY101 upon treatment with dexamethasone. The synthetic glucocorticoid, able to bind and induce translocation of the GR into the nucleus, triggered the expression of TEV and as consequence degradation of γ EGFP. As expected tdTandem tomato fluorescence did not changed upon hormone treatment (data not shown).

Once the appropriate response of the strain RVY101 to dexamethasone was confirmed, in order to complete the construction of the assay strain RVY102, the expression vector carrying the construct *GAL1-HSD11B1* was transformed in RVY101. As previously confirmed in the strain RVY97, the *GAL1* promoter allowed a fine and simultaneous expression of the 11 β -HSD1 together with GR and a constant expression of the N-Degron fluorescent reporter protein.

Functionality of the assay strain RVY102 was tested by FACS analyses. The green fluorescence response was monitored at different time points upon addition of different cortisone concentrations (from 125 μ M to 2000 μ M) (**Fig. 4.18a**). As expected, treatments with increasing concentrations of cortisone caused a gradual loss of γ EGFP after 6 and 9 hours of incubation, indicating the reduction of cortisone to cortisol driven by 11 β -HSD1 and the consequent expression of TEV protease. Conversely, cells treated with DMSO maintained stable expression levels of the fluorescent fusion protein. Significant γ EGFP degradation was already observed upon 6 hours of treatment with 1000 and 2000 μ M of cortisone and degradation further increased upon 9 hours of incubation (**Fig. 4.18a**). Fluorescence microscopy and FACS measurements also showed the decrease in γ EGFP signal, denoting a loss of green fluorescence upon cortisone treatment whereas td-Tomato fluorescence remained constant (**Fig. 4.18, b and c**).

To select for the best experimental conditions many factors including cortisone solubility and toxicity, time of incubation, and fluorescent response were experimentally evaluated. Finally, 6 hours of incubation with 1000 μ M cortisone were employed for the validation of strain RVY102.

The specificity of the signal upon treatment with cortisone was confirmed with a strain in which the plasmid, carrying the 11 β -HSD1 expression cassette, has been replaced by the equivalent empty

plasmid. The strain was, except for the missing 11 β -HSD1 expression construct, identical to the parental assay strain RVY102. No response to cortisone was detected in the control strain.

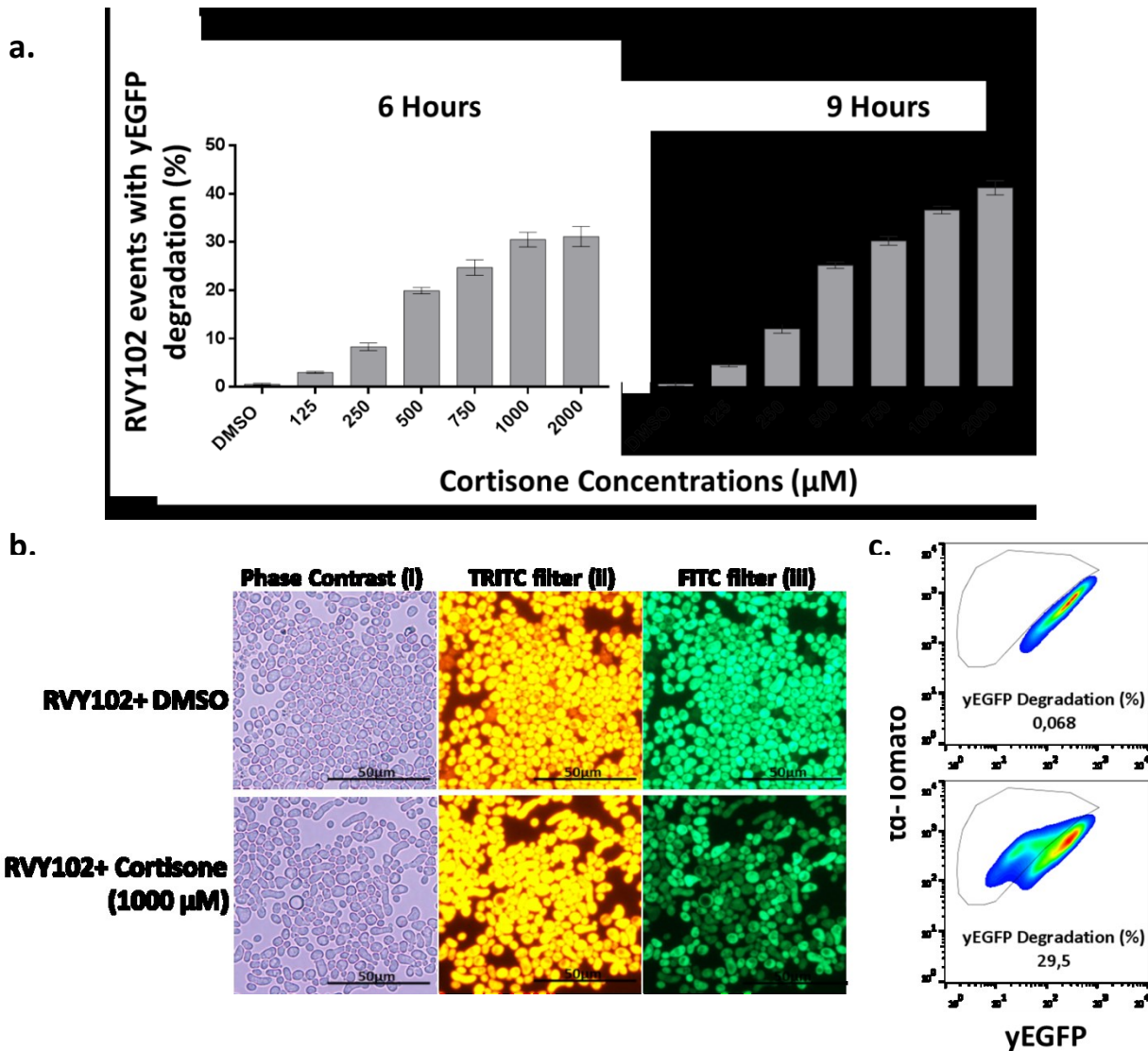


Fig.4.18. Effect of cortisone on the yEGFP degradation in yeast strain RVY102. **a)** Increased cortisone concentrations caused the expression of the TEV protease in a time and concentration dependent manner. The TEV protease acted on the reporter protein construct causing degradation of the yEGFP. At all-time points tested the yEGFP degradation signal in the cortisone-treated cells was significant with respect to the signal of the negative control cells treated with DMSO. **b)** RVY102 yeast population treated with DMSO and 1000 μM cortisone. Pictures acquired 6 hours upon initiation of the treatment with cortisone showed degradation of yEGFP in part of the population (iii), while the tdTomato signal remained constant in both treated and untreated cells (ii). **c)** FACS analysis of RVY102 strain treated for 6 hours with DMSO (upper panel) and 1000 μM cortisone (lower panel). Cells that show yEGFP degradation are gated.

In order to validate the yeast strain RVY102 for screening of putative 11 β -HSD1 inhibitors, carbenoxolone concentrations ranging from 0.1 to 3 μ M were added to 1 million RVY102 cells together with 1000 μ M cortisone. FACS analyses revealed that increasing concentrations of carbenoxolone gradually led to an increase of the reporter protein in the population. At a concentration of 3 μ M carbenoxolone almost no degradation of γ EGFP was detected suggesting complete inhibition of the enzyme 11 β -HSD1 (Fig. 4.19).

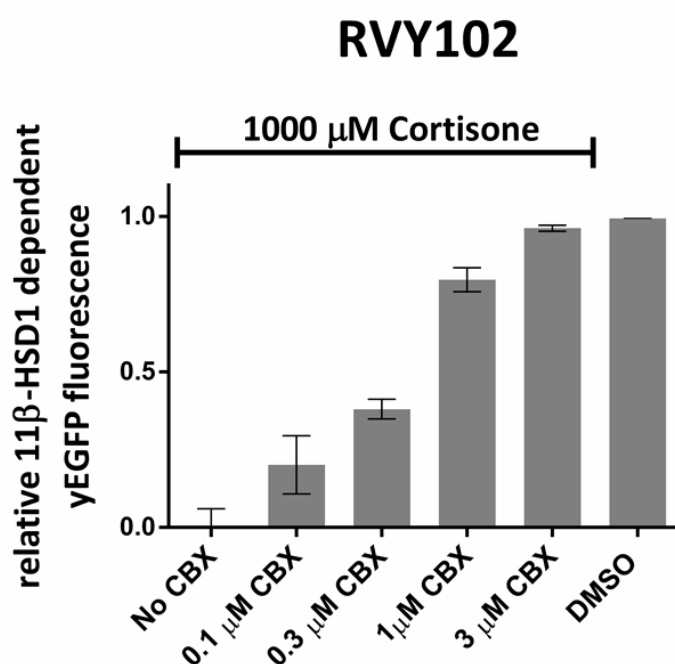


Figure 4.19. 11 β -HSD1 dependent γ EGFP fluorescence of strain RVY102 treated with 1000 μ M cortisone and increasing CBX concentrations. Increasing concentration of carbenoxolone improved the stability of γ EGFP in the yeast population indicating a progressive loss of enzyme activity. Cells treated with 1000 μ M cortisone and 3 μ M carbenoxolone showed a fluorescence comparable to the one of the untreated population (DMSO) indicating a complete absence of γ EGFP degradation. tdTomato fluorescence was maximum and constant in all samples analysed and was not affected by treatments with cortisone and carbenoxolone. For data normalization details see “Experimental procedures”.

Improving the sensitivity of the assay systems by inhibiting Pdr5p

The relatively high concentration of cortisone required to trigger the yeast based assays, when compared to the concentration of carbenoxolone needed to completely inhibit 11 β -HSD1 raised the question whether the influx of cortisone might be limited, and therefore intracellularly the cortisone concentration is much lower than expected. Consequently, the intracellular availability of cortisone was investigated.

Previous studies demonstrated that in yeast, a low accumulation of certain steroid molecules due to the action of membrane-embedded drug-efflux pumps, can strongly affect the functionality of an intracellular cascade driven by the heterologous GR-hormone complex (Wright et al., 1990; Kralli et al., 1995). In the yeast *Saccharomyces cerevisiae*, an ATP-binding cassette (ABC) transporter, Pdr5p, is able to decrease accumulation of certain steroids, thus changing cellular responses caused by GR-ligand complexes. Pdr5p has many different steroid and non-steroid substrates and tends to protect the cells from an improper accumulation of undesired molecules (Mahé et al., 1996; Kolaczowski et al., 1996; Rogers et al., 2001). The null mutants of this transporter allow accumulation of glucocorticoids in cells and maximal activation of the heterologous GR. There is evidence that dexamethasone, a synthetic equivalent of cortisol, is an important substrate of the transporter Pdr5p (Kralli et al., 1995). Therefore, a possible recognition and excretion of cortisone by Pdr5p was investigated. The expression and activity of Pdr5p in strains RVY97 and RVY102, respectively, was studied upon treatment with 5 μ M Rhodamine 6G, a well-known target of Pdr5p (Kolaczowski et al. 1996). Through accumulation of this specific dye within yeast cells, it is possible to get indications about the activity of this transporter. To confirm that accumulation of Rhodamine 6G is limited due to Pdr5p activity, the Pdr5p inhibitor FK506 (Kralli et al., 1996) was added to the cells leading to a strong intracellular accumulation of the dye (**Fig. 4.19**). Those experiments demonstrated the presence of significant amounts of active transporter molecules Pdr5p in the assay strains.

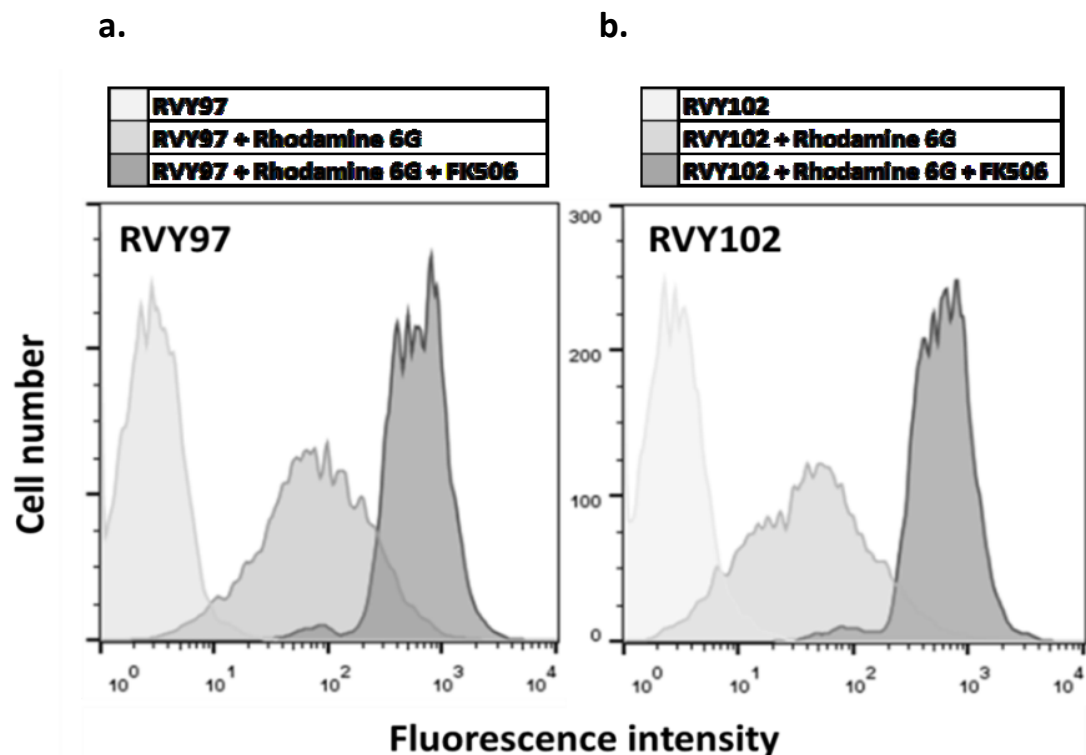
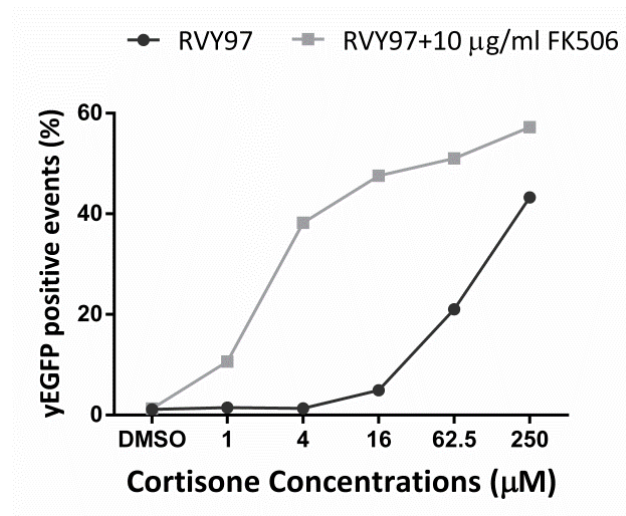


Figure 4.20. Rhodamine 6G accumulates in the cells upon Pdr5p inhibition. Rhodamine 6G accumulation in **a)** RVY97- and **b)** RVY102- yeast strains treated or not treated with 10 $\mu\text{g/ml}$ FK506, a known inhibitor of Pdr5p.

Next, the green fluorescent output of strains RVY97 and RVY102 was examined in the presence of 10 $\mu\text{g/ml}$ FK506 and upon treatment with different concentrations of cortisone. Addition of FK506 dramatically improved the sensitivity of the assay strains to cortisone. In fact, hormone concentrations up to 60 times lower than used before caused equivalent responses of the RVY97 and RVY102, when the Pdr5p transporter was not active (**Fig. 4.20**). These results strongly suggested that cortisone is a substrate of the Pdr5p transporter.

a.



b.

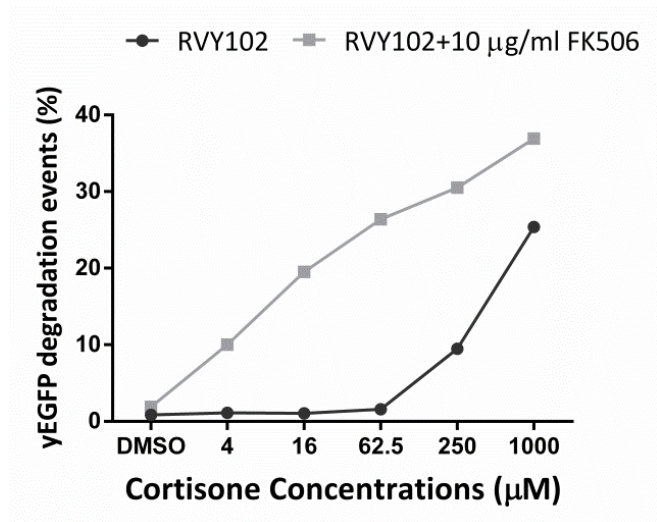


Figure 4.21. Effects of FK506 on RVY97 and RVY102 assays functionality. Both **a)** RVY97 and **b)** RVY102 assays have been tested with or without FK506 at different concentrations of cortisone. In both assays an increased sensitivity to cortisone in presence of the Pdr5p inhibitor is shown. Each experiment was performed at least three independent times with similar results. The figure represents a single result of the replicates.

5

Conclusions

Intracellular glucocorticoid levels play a relevant role in the pathogenesis of metabolic diseases such as obesity and metabolic syndrome. Moreover, in the last years, high levels of active glucocorticoids in skin have been associated with impaired collagen metabolism and reduced wound healing processes in diabetic and elderly individuals. Therefore, inhibitors of 11 β -HSD1, the main enzyme responsible for the activation of glucocorticoids at intracellular level, have been proposed as crucial compounds for the treatment of these conditions and for development of skin care products that regulate skin homeostasis. Many strategies to find specific inhibitors for this enzyme have been developed, but only a few molecules have been identified. Among other reasons, this might be due to the fact that the screening of large libraries of small molecules and the identification of pharmaceutically active candidates need very efficient assay systems, often difficult to establish, or whose development is very cost- and labour intense.

The research work presented in this thesis casts the first stone for the realization of a new approach for synthesis and screening of inhibitors of the human enzyme 11 β -HSD1. The original idea was born from collaboration with an industrial partner, Evolva SA, and intends to use yeast cells in order to identify new specific compounds for the pharmacological inhibition of the isozyme 11 β -HSD1, exploiting Evolva SA's Synthetic Biology Technology Platform. This platform couples combinatorial expression of multiple genes, from plant or other organisms, in the yeast *Saccharomyces cerevisiae* with screening systems that can identify compounds with the desired properties.

In order to reach the final step of production and screening of compounds, this work experimentally investigated the fundamental aspects for the success of the entire strategy.

Triterpenoids are a large class of natural secondary metabolites mainly represented in plants. Due to their well-known positive effects on human health they are often used as a source for compounds with pharmaceutical applications. Moreover, components of this family are known to exert inhibitory effects against the human enzyme 11 β -HSD1, but in some cases, due to their low selectivity towards the 11 β -HSDs, they cannot be intensively used for treatments.

Since triterpenoids represent a promising source of new inhibitors of 11 β -HSD1, this study aimed at the reconstruction of their biosynthetic pathway in yeast through expression of different classes of natural triterpenoid synthases. Moreover, additional genetic information, in order to provide new enzymatic activities and to further increase the chemical scaffold diversity synthesized in yeast, were generated by gene shuffling procedures, applied to groups of triterpenoid synthase cDNAs. Among many methods for random mutagenesis, two DNA family shuffling protocols were selected for the construction of the final library. The original DNA family shuffling method developed by Cramer and

colleagues in 1998, was successfully adapted and applied. Nevertheless low numbers of recombination events, high percentages of random point mutations and strong presence of wild-type parental sequences were usually detected in the final mixture of molecules.

The Synthetic Biology Technology Platform investigated here intends to use a multiple cloning strategy for simultaneous expression of the new genes in yeast in order to screen for compounds with 11 β -HSD1 inhibitory activity. The strong presence of unmodified triterpenoid synthase cDNAs obtained from the application of the original DNA family shuffling method could dramatically lower the number of chimeric sequences represented in the assembled expressible yeast artificial chromosomes. In order to guarantee a higher presence of mutant triterpenoid cyclase cDNAs and to reduce the contamination with parental unmodified cDNAs, a new DNA family shuffling method, defined as semi-synthetic, was developed. Although this method did not provide a random fragmentation of sequences, it guaranteed a highly efficient recombination process during the primerless PCR. Results in fact demonstrated the absence of parental non-modified genes and constant high numbers of recombination events in the final mixture of sequences. Through this method a large library of about 8-9 millions of new sequences, potentially codifying for chimeric triterpenoid synthases, was constructed.

The library of chimeric DNA sequences assembled during the first part of the work as well as a library of parental triterpenoid synthase cDNAs were employed for construction of eYAC molecules. eYACs were assembled with the genetic information that provided the yeast with the missing functions for the synthesis of plant triterpenoids. The expression of natural triterpenoid synthases aimed at creating natural triterpenoid scaffolds that represent an important source of pharmaceutical active compounds. In addition, the simultaneous expression of large numbers of chimeric triterpenoid synthases aimed at providing more chemical variability among the synthesized molecules. The eYACs construction protocol originally conceived at Evolva SA, was adapted and applied to the purposes of this work in order to reach the best outcome. However, due to the significant presence of similar sequences in the DNA mixture employed for preparation of the chromosomes, spontaneous recombination processes caused instability of the final molecules. To overcome this problem, different concentrations of non-functional exogenous DNA were added to the mixture of triterpenoid synthase expression cassettes. The external DNA material diluted the concentration of similar sequences involved in the concatenation, providing higher stability to the chromosomes. eYACs were successfully transformed in yeast in order to study the production of triterpenoids and to test for the expression and functionality of the synthases. After appropriate cultivation, the organic extracts of yeast populations carrying eYACs were analysed through GC-MS and no naturally occurring

triterpenoid molecules were detected in any of the samples. This result could be due to the impaired expression or functionality of the wild type triterpenoid synthases yielding a production level below the limit of detection.

In order to investigate the functionality of parental and chimeric triterpenoid synthases, a group of parental β -amyrin synthases and twenty chimeric triterpenoid synthases were individually expressed in yeast and the organic extracts were analysed through GC-MS. The expression of two of four parental β -amyrin synthases led to detectable production of β -amyrin. Moreover, functionality was confirmed for three of the twenty chimeric enzymes expressed. These data therefore suggested that most likely the absence of detectable triterpenoids in the yeast carrying eYACs was due to the short size of the chromosomes representing only a limited number of expression cassettes and not to the impaired functionality of the enzymes expressed. Nevertheless a wider screening of yeast clones carrying eYACs would be necessary to confirm this assumption.

CG-MS analyses generally provide a useful strategy for triterpenoid detection in yeast. However the method applied, alone, was not advanced enough to screen for new chemical scaffolds produced by the activity of chimeric enzymes. In fact, since no predictions could be made on the possible structures to be detected, more complex approaches such as differential analysis, liquid chromatography-mass spectrometry, derivatization of compounds etc., would be needed.

In future, the stability and the quality of the eYACs will be certainly increased by adding a library of cDNAs codifying for decorating enzymes such as glycosyltransferases, sulfotransferases, transaminases, acyl-transferases and cytochrome P450 oxygenases, currently under construction at Evolva, to the other genetic information used for the construction of the chromosomes. These additional cDNAs will replace the non-functional DNA used during this work to dilute the concentration of similar sequences in the eYACs and will also provide new genetic information for classes of enzymes that can add modifications to the chemical scaffolds, most likely leading to a dramatic increase of the chemical diversity synthesized.

Production of natural and new chemical scaffolds by the yeast cells carrying the new assembled chromosomes will be directly screened through *in vivo* assays for the identification of molecules with inhibitory activity towards 11 β -HSD1.

In order to develop functional yeast-based assays for screening of molecules with 11 β -HSD1 inhibitory activity, many efforts were dedicated during the work to the construction and validation of two yeast strains. Both strains, RVY97 and RVY102 functionally connect the reductase activity of the human enzyme to a fluorescent output. The two assay-systems were successfully tested and functionally evaluated upon treatments with different cortisone concentrations. The specificity of the

fluorescent response was demonstrated through development of negative control strains identical to the originals but lacking the 11 β -HSD1 construct, which did not respond to cortisone treatments. Moreover, the assays have been validated using carbenoxolone, one of the most widely used 11 β -HSD1 inhibitors. The results demonstrated a clear connection of the fluorescent outputs with the reductase activity of the 11 β -HSD1 enzyme. Increasing carbenoxolone concentrations caused changes in the fluorescent signal of both strains indicating inhibition of 11 β -HSD1 and the results were reproducible and comparable between the two independent strains. Finally, the sensitivity of the yeast based assays to cortisone was significantly improved through inhibition of the yeast multidrug transporter Pdr5p. Cortisone was demonstrated to be an effective substrate of this efflux pump that usually prevents its accumulation at intracellular level.

The developed yeast strains will be used for transformation of new eYAC molecules and screening for inhibitors of 11 β -HSD1. The fluorescent output connected with the activity of the enzyme can allow for selection of single cells producing inhibitors. The positive hits are then isolated through fluorescence activated cells sorting and further investigated to identify the active compounds.

The yeast-based screening systems described here represent also a valid alternative to currently available assays for the screening of 11 β -HSD1 inhibitors. Through the addition of the compounds to be tested in the growth medium, both RVY97 and RVY102 strains are efficient and adaptable systems for the screening and selection of cell permeable compounds with inhibitory activity, before progressing to expensive, low-throughput and time-consuming *in vivo* animal studies.

The construction of the yeast strain RVY97 and RVY102 and their validation are object of a scientific publication currently under submission. Both strains and their assay mechanisms are protected by a patent.

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Future Perspectives

The yeast-based platform investigated in this thesis intends to use the developed yeast strains for a single cell screening process in order to isolate events that produce 11 β -HSD1 inhibitors. After eYACs preparation the yeast transformants will be cultivated in liquid media and the single cells screened by FACS upon treatment with cortisone. The positive hits (no fluorescent RVY97- and green fluorescent RVY102- cells) that indicate inhibition of the human enzyme are then sorted and further investigated in order to isolate and identify the active compounds.

RVY97 and RVY102 respond properly to the treatment with cortisone, in fact the overall fluorescence output detected from the population indicates activity of the 11 β -HSD1 enzyme on the hormone. Reversely, in presence of cortisone and carbenoxolone, a known 11 β -HSD1 inhibitor, the output detected from the populations indicates inhibition of 11 β -HSD1.

Even though all the analyses conducted in this work were performed through FACS, the assays functionality was tested looking at the overall response of the isogenic population and not at the signal of single cells. Nevertheless, FACS analysis clearly led to the evidence that both isogenic populations, RVY97 and RVY102, do not respond uniformly to the treatment with the hormone.

The background signal that results upon cortisone treatment does not influence the use of the two systems if the candidate inhibitors are added into the growth medium, since the response is detected from the entire population. However, the heterogeneous response would have important effects on the single cell screening mode that the final platform intends to use, thus, causing the presence of many false-positive events. For this reasons, further optimizations on the yeast strains developed are needed to ensure a more uniform response of the populations to cortisone and consequently to lower the presence of false positives.

The heterogeneous response in a yeast isogenic population has been largely studied in the past and, among many possible causes, the non-uniform inheritance of the two 2 μ plasmids used for the strains construction is most likely the origin of the not homogenous response. Both RVY97 and RVY102, respectively, contain two 2 μ plasmids carrying 11 β -HSD1 and GR expression cassettes. The amount and ratio of these two mammalian proteins need to be uniform and constant within the cells of an isogenic population in order to efficiently cooperate and trigger the downstream cascade in every single cell.

The use of the ectopic 2 μ plasmids rather than the genomically integrated constructs was chosen in order to maximize the expression of the two mammalian proteins, essential for the response of the yeast strains to the treatments with the hormone. In fact, this was the only strategy that allowed a proper response of the strains to the treatments with cortisone.

During the work, it has been demonstrated that the sensitivity of the developed assays to cortisone improves significantly when *Pdr5p* is inhibited, due to the higher intracellular levels of cortisone.

Due to this evidence, *Pdr5* gene deletion might allow for a significant response of the developed strains to cortisone even in presence of lower levels of 11 β -HSD1 and GR expressed. For this reason, the single or multi-copy integration of the 11 β -HSD1 and GR expression constructs in the genome, that would lead to lower levels of the mammalian proteins in the yeast cells but to higher genetic uniformity, may be, together with the *Pdr5* gene deletion, the best strategy to achieve both, a good sensitivity of the systems and a reduction of the background signal upon cortisone treatments in the final assay strains.

In order to increase the amount of triterpenoids synthesized in the yeast assay strains and thus to enhance the possible effects of these molecules towards the enzyme 11 β -HSD1, the developed strains could be functionally re-engineered. Downregulation of the *erg7* gene, responsible for the expression of the yeast lanosterol synthase, would lead to accumulation of 2,3 epoxy-squalene within the yeast cells. This metabolite, the common precursor to all the triterpenoids, can strongly promote the cyclization process driven by the plant and chimeric triterpenoid synthases expressed in yeast thus increasing the production of triterpenoids within the single cells.

Before progressing to the final eYACs preparation and screening, an important experiment to perform can be the reproduction of a biosynthetic pathway for a known 11 β -HSD1 inhibitor in the yeast assay strains. This will provide important information about the overall mechanisms of the screening strategy here investigated and can be used as positive control strain for the final screening procedure. Some efforts were spent in this work for the reconstitution of the biosynthetic pathway of glycerretinic acid in the RVY97 and RVY102 strains. Despite the presence of all the genetic information to reproduce the pathway, in a first analysis, the synthesis of the glycerretinic acid was not detected.

Finally, although still some work needs to be done, the results of this PhD thesis strongly support the initial innovative and challenging idea for production and screening of novel 11 β -HSD1 inhibitors.

I believe that this work can introduce in the scientific community some innovative concepts and information, and can be a source of inspiration for advances in drug-discovery processes.

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Experimental Procedures

All the PCRs, restriction reactions and ligations performed in this work followed standard protocols whether not otherwise specified.

Triterpenoid synthase cDNAs cloning

The triterpenoid synthase cDNAs (**Table 4.1**) have been codon optimized for expression in yeast *S. cerevisiae* and provided by Evolva. All cDNAs were cloned without stop codon and in frame with a sequence coding for a polyhistidine tag in the vector pET300 CT-His, prior to be used for the application of the DNA family and semi-synthetic shuffling methods. Bacterial cells containing the plasmids carrying the cDNAs were inoculated in 3 ml of Luria Broth medium with 100 µg/ml Ampicillin and incubated overnight at 37°C shaking at 180 rpm. Small scale preparation of DNA plasmid was performed with the PureYield Plasmid Miniprep System kit following manufacturer's instructions. The final DNA concentration was measured with NanoDrop 2000c spectrophotometer. Based on the similarity values of the triterpenoid synthase cDNAs, three groups of sequences were selected for the application of the shuffling methods (**Fig. 4.1**). Each group consisted of 4 different cDNAs sharing between 73% and 85% of the nucleotide sequences.

DNA family shuffling

The original DNA family shuffling protocol (Cramer et al., 1998) was adapted in order to obtain the best experimental outcome. Each cDNA molecule was initially PCR amplified from the recipient vector pET300 CT-His in order to collect large amount of material. The PCR was carried out with universal external primers T7 promoter and T7 reverse (**Table 7.1**) at a concentration of 0.5 µM each, together with 1 unit Phusion HF DNA Polymerase, 1x Phusion HF Buffer, 200 µM of each dNTP and 1-5 ng of template in a total volume of 20 µl. The PCR program employed consisted of 95°C for 5 min as initial step, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 3 min. A final elongation step of 5 min at 72°C was performed. PCR products were analysed on a 1% (w/v) agarose gel with 1 µg/ml ethidium bromide and visualized with an UV-transilluminator. All DNA products were individually purified from agarose gel through NucleoSpin Extract II kit according to manufacturer's instructions and the concentration was

measured using NanoDrop 2000c. According to the original protocol, an equimolar mixture (1-4 μ g) of molecules to treat together was set before to proceed with the DNaseI reaction.

The DNA equimolar mixture was digested with 0.0075 U of DNaseI at 25°C for 4-10 min. The reaction mixture was completed with 1x DNaseI buffer in a total volume of 40 μ l. After incubation the reaction was stopped with high excess of EDTA (final concentration: 20 mM) and loaded in a 2% (w/v) agarose gel for DNA electrophoresis (20 min at 130 Volts). DNA fragments between 30 and 150 bp were purified from agarose gel with NucleoSpin Extract II kit according to manufacturer's instructions and employed for the subsequent reassembling PCR.

DNA fragments (15ng/ μ l) were prepared in a reaction mixture together with 1U Phusion HF DNA polymerase, 1x Phusion HF Buffer and 200 μ M of each dNTP. The recombination PCR was carried out using the following conditions: first denaturation step at 95°C for 1 minute and then 50 cycles of 95°C for 30 seconds, 52°C for 1 minute, 72°C for 2 minute followed by a final amplification step at 72°C for 10 min. Part of the PCR product was loaded on a 1% (w/v) agarose gel, stained with ethidium bromide 1 μ g/ml and visualized under UV light.

Full length chimeric sequences were amplified using as template the primerless PCRs product at different dilutions. Amplifications were carried out under the following conditions: one step of 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 2.30 min. A final elongation step at 72°C for 5 min was performed. Each reaction consisted of 1 unit FIREPol DNA Polymerases, 1x reaction Buffer B, 2.5 mM MgCl₂, 200 μ M each dNTP, 0.5 μ M of each primer and as template a dilution of the primerless PCR product, in a total volume of 20 μ l. Typically, several reactions with different template dilutions were performed to ensure the best amplification results. The products were verified on a 1% (w/v) agarose gel with 1 μ g/ml ethidium bromide. The right size DNA bands (2.3 kb) were purified using NucleoSpin Extract II kit.

DNA semi-synthetic shuffling

Primer designing

According to the sequences alignment in every group of cDNAs 10 conserved regions were identified and selected for common primers designing. Right and left overlapping primers of 10-15 bp were drawn to bind all four genes of the same group in the same regions. 10 couple of primers were designed for each group of genes (**Table 7.1**).

Table 7.1. List of oligonucleotides employed in the DNA semi-synthetic Shuffling method.

Primer Name	Sequence (5'→ 3')	Application
EV47484950 For1	CAAACATGGGAGTTC	Shuffling group I
EV47484950 Rev1	GAACTCCCATGTTTG	Shuffling group I
EV47484950 For 2	GATGGACACTGGCCTGC	Shuffling group I
EV47484950 Rev 2	GCAGGCCAGTGTCCATC	Shuffling group I
EV47484950 For3	ATGAGGATCTTGGGAG	Shuffling group I
EV47484950 Rev3	CTCCCAAGATCCTCAT	Shuffling group I
EV47484950 For4	CCAGCAAAGATGTGGTG	Shuffling group I
EV47484950 Rev4	CACCACATCTTTGCTGG	Shuffling group I
EV47484950 For5	AGATGGCCATTCAACAA	Shuffling group I
EV47484950 Rev5	TTGTTGAATGGCCATCT	Shuffling group I
EV47484950 For6	ATGCAATCTTTCGGT	Shuffling group I
EV47484950 Rev6	ACCGAAAGATTGCAT	Shuffling group I
EV47484950 For7	GATCATGGATGGCAAGT	Shuffling group I
EV47484950 Rev7	ACTTGCCATCCATGATC	Shuffling group I
EV47484950 For8	GAATGGTTGGAGCTTCTTAA	Shuffling group I
EV47484950 Rev8	TTAAGAAGCTCCAACCATTC	Shuffling group I
EV47484950 For9	TGGTACGGCAATTGGGG	Shuffling group I
EV47484950 Rev9	CCCAATTGCCGTACCA	Shuffling group I
EV47484950 For10	CCATTACACAGGGCTGC	Shuffling group I
EV47484950 Rev10	TGCTGCCCTGTGTAAAGG	Shuffling group I
EV525354C405 For1	TGGGAGTTCGACCCT	Shuffling group II
EV525354C405 Rev1	AGGGTCGAACTCCCA	Shuffling group II
EV525354C405 For2	GATGGACATTGGCCTGG	Shuffling group II
EV525354C405 Rev2	CCAGGCCAATGTCCATC	Shuffling group II
EV525354C405 For3	CTAATGATGGGGAAGG	Shuffling group II

EV525354C405 Rev3	CCTTCCCATCATTAG	Shuffling group II
EV525354C405 For4	CCAGGTAGGATGTGGTG	Shuffling group II
EV525354C405 Rev4	CACCACATCCTACCTGG	Shuffling group II
EV525354C405 For5	CATGATATTGATTGGAA	Shuffling group II
EV525354C405 Rev5	TTCCAATCAATATCATG	Shuffling group II
EV525354C405 For6	ATGTTGTGTTGTTGGGT	Shuffling group II
EV525354C405 Rev6	ACCCAACAACACAACAT	Shuffling group II
EV525354C405 For7	CACGGCTGGCCAAT	Shuffling group II
EV525354C405 Rev7	ATTGCCAGCCGTG	Shuffling group II
EV525354C405 For8	CCTGCTGAGACTTTTGG	Shuffling group II
EV525354C405 Rev8	CCAAAAGTCTCAGCAGG	Shuffling group II
EV525354C405 For9	TGGGGAGTCTGCTT	Shuffling group II
EV525354C405 Rev9	AAGCAGACTCCCCA	Shuffling group II
EV525354C405 For10	AACACAGGCTGGGCTATG	Shuffling group II
EV525354C405 Rev10	CATAGCCCAGCCTGTGTT	Shuffling group II
EV42434849 For1	TCTACTAACAACTT	Shuffling group III
EV42434849 Rev1	AAGTTGTTAGTAGA	Shuffling group III
EV42434849 For2	CTACAGGCAAGTGATGG	Shuffling group III
EV42434849 Rev2	CCATCACTTGCCTGTAG	Shuffling group III
EV42434849 For3	ACATATATTGTCATCAAAA	Shuffling group III
EV42434849 Rev3	TTTTGATGACAATATATGT	Shuffling group III
EV42434849 For4	TGGGGTAAACCTGG	Shuffling group III
EV42434849 Rev4	CCAGGTTTTACCCCA	Shuffling group III
EV42434849 For5	ATGAGTTATCTTTA	Shuffling group III
EV42434849 Rev5	TAAAGATAACTCAT	Shuffling group III
EV42434849 For6	CCTTTACTTACTAGAT	Shuffling group III
EV42434849 Rev6	ATCTAGTAAGTAAAGG	Shuffling group III
EV42434849 For7	GACTTTAGAAGTATGT	Shuffling group III
EV42434849 Rev7	ACATACTTCTAAAGTC	Shuffling group III
EV42434849 For8	AAGTGCTGTTTACTTTT	Shuffling group III
EV42434849 Rev8	AAAAGTAAACAGCACTT	Shuffling group III
EV42434849 For9	TGGTATGGCAATTGGGG	Shuffling group III
EV42434849 Rev9	CCCAATTGCCATACCA	Shuffling group III
EV42434849 For10	CAAGCCGAAAGAGATC	Shuffling group III
EV42434849 Rev10	GATCTCTTTCGGCTTG	Shuffling group III
T7 promoter	TAATACGACTCACTATAGGG	Upstream Universal
T7 reverse	TAGTTATTGCTCAGCGGTGG	Downstream Universal

Fragments amplification

Eleven fragments from each sequence were amplified by PCR. The typical length of these fragments ranged between 100-500 bp. The amplification mix consisted of 1 Unit of FIREPol DNA Polymerase, 1x reaction Buffer B, 2.5 mM MgCl₂, 200 μM of each dNTP and 0.5 μM of each primer in a total volume of 20 μl. As template 1-5 ng of the corresponding cDNA sequence was added to the reaction mixture. PCR conditions were as follows: one step of 95°C for 5 min, 35 cycles of 95°C for 30 seconds, 52°C for 30 seconds and elongation at 72°C for 20 seconds. Finally an elongation step of 5 min at 72°C was performed. In the case of low quality results the PCR was repeated decreasing the annealing temperature to 38°C. PCR products were analysed on a 1.5% (w/v) agarose gel with 1 μg/ml ethidium bromide and visualized with an UV-transilluminator. All DNA fragments were individually purified from agarose gel using NucleoSpin Extract II kit according to manufacturer's instructions and the DNA concentration measured with NanoDrop 2000c.

Primerless PCRs

The amplified fragments were used as unique DNA material in an overlap extension PCR. Each group of fragments amplified with the same primers and belonging to the four different cDNAs of the same group were organized in equimolar mixtures of 70 ng and added to the reaction. The final amount of DNA fragments in the reaction was 770 ng in a total volume of 50 μl. The reaction mixture was completed with 1 unit Phusion HF DNA Polymerase, 1x Phusion HF Buffer and 200 μM of each dNTP. Recombination PCR was carried out using the following conditions: first denaturation step at 95°C for 1 minute and then 50 cycles of 95°C for 30 seconds, 52°C for 1 minute, 72°C for 1 minute followed by a final amplification step at 72°C for 5 min. Afterwards 10 μl of the PCR product were loaded on a 1% (w/v) agarose gel stained with 1 μg/ml ethidium bromide and visualized under UV light. 25 μl from the first recombination PCR were used as template in a second primerless PCR. 1 unit of Phusion HF DNA Polymerase, 1x Phusion Buffer and 200 μM each dNTP were added to 25 μl of the previous reaction in a final volume of 50 μl. PCR conditions for the second recombination PCR consisted of 95°C for 5 min as initial step, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute. A final elongation step of 5 min at 72°C was added. 10 μl of the PCR product were verified on a 1% (w/v) agarose gel with 1 μg/ml ethidium bromide and visualized with an UV-transilluminator.

Full-length chimeric cDNAs amplification

As final step of the DNA semi-synthetic shuffling protocol, the full length chimeric sequences were amplified from the Primerless PCR products. For each group of parental cDNAs, left and right primers binding upstream and downstream of all the four different sequences were designed (**Table 7.2**). *HindIII* and *SacII* restriction sites were added to the forward and reverse primers, respectively, in order to perform further cloning steps. Amplifications were carried out at the following conditions: one step of 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 2.30 min. A final elongation at 72°C for 5 min was performed. Each reaction consisted of 1 U FIREPol DNA Polymerases, 1x reaction Buffer B, 2.5 mM MgCl₂, 200 μM each dNTP, 0.5 μM of each primer and as template serial dilutions of the second primerless PCR product, in a total volume of 20 μl. Typically, several reactions with different template dilutions were performed to ensure the best results. The products were verified on a 1% (w/v) agarose gel with 1 μg/ml ethidium bromide. The right size DNA bands (2.3 kb) were excised and purified using NucleoSpin Extract II kit.

Table 7.2. Primers used in the final chimeric cDNA amplification of the semi-synthetic DNA Shuffling method.

Primer Name	Sequence (5' → 3')	Application
UniversalRevSacII	GCATACCCGCGGTCAGTGGTGGTGGTGGTGGTG	Final PCR
ForEv47HindIII	GCATACAAGCTTAAAATGTGGAAATTGAAAATTGG	Final PCR
ForEv48HindIII	GCATACAAGCTTAAAATGTGGAGATTGAAAATTGC	Final PCR
ForEv49HindIII	GCATACAAGCTTAAAATGTGGAAATTGAAAATTGC	Final PCR
ForEv50HindIII	GCATACAAGCTTAAAATGTGGAGATTGATGACTGC	Final PCR
For525354HindIII	GCATACAAGCTTAAAATGTGGAAATTGAAAATTGC	Final PCR
ForC405HindIII	GCATACAAGCTTAAAATGTGGAAAGCTCAAGGTTGC	Final PCR
ForEV42HindIII	GCATACAAGCTTAAAATGTGGAAATTGAAAATTGC	Final PCR
ForEV43HindIII	GCATACAAGCTTAAAATGTGGAGAATTAATAATTGC	Final PCR

cDNA library construction

The mixture of chimeric cDNAs obtained from the application of the DNA semi-synthetic shuffling was cloned into entry vectors for the eYACs construction. The same procedure in parallel was performed for the ten parental cDNA molecules (**Table 4.1**) used as starting material in the DNA shuffling protocols.

Double digestion of chimeric sequences and entry vectors

The mixture of sequences obtained from the mutagenesis protocol and each destination entry vector were double digested, in separate reactions, with *Hind*III and *Sac*II enzymes. The reaction typically consisted of 1-2 µg of DNA, 1x Cutsmart Buffer and 10 U of each restriction enzyme in a total volume of 50 µl. Plasmids digestion was usually performed for 2 hours at 37°C. The reaction was verified through DNA electrophoresis and the right size band was purified from agarose gel using NucleoSpin Extract II kit. The mixture of chimeric DNA was digested at 37°C overnight and the products purified directly from the reaction mixture with NucleoSpin Extract II kit.

Cloning of chimeric products into the entry vectors

The mixture of genes obtained from the DNA semi-synthetic shuffling, after digestion, was cloned separately into six destination entry vectors (**Table 7.3**). All ligation reactions consisted of 400 U of T4 DNA ligase (NEB), 1x T4 DNA ligase Buffer in a final volume of 10 µl or 20 µl. 20 ng of digested vector were used as destination recipient plasmid and according to the ratio 1:5 (vector: insert) a certain amount of digested DNA insert was added to each reaction. All the ligation reactions were performed at 4°C for 8 hours followed by a step of 6 hours at 16 °C. After ligation the DNA was purified from the reaction with NucleoSpin Extract II kit and used to transform chemically competent high efficiency *E.coli* NEB 5-alpha cells according to manufacturer's instructions. Transformed cells were plated on LB agar plates supplemented with Ampicillin 100 µg/ml for selection of positive clones. Several ligation processes and transformation steps were performed during the work in order to obtain a high number of colonies and representation of high numbers of chimeric cDNAs in the final library. After every transformation process the colonies were collected from the agar plates and conserved in a final solution of 50% glycerol at -80°C. Exactly the same procedure was used to construct the

library of parental cDNAs in the recipient entry vectors. Colony PCR procedures and restriction reactions of random purified plasmids were used to assess the quality of the final cDNA libraries.

Table 7.3. List of entry vectors used as recipient plasmids for the chimer triterpenoid synthases and parental cDNAs. In table are indicated yeast promoter and terminator contained in each vector. A schematic map of these vectors can be visualized in figure 4.7.

Vector	Yeast Promoter	Yeast Terminator	Application
pEVE3591	pTDH3	CYC1t	cDNA library and eYACs construction
pEVE3611	pADH1	PGK1t	cDNA library and eYACs construction
pEVE3612	pPGK1	ADH2t	cDNA library and eYACs construction
pEVE3613	pPYK1	TEF1t	cDNA library and eYACs construction
pEVE3614	pTEF1	ENO2t	cDNA library and eYACs construction
pEVE3618	pTPI1	TDH1t	cDNA library and eYACs construction

To confirm the success of the DNA semi-synthetic shuffling method applied, more than 60 new DNA molecules obtained through shuffling and represented in the cDNA library, were sent for sequencing to Microsynth AG. Results were analysed and compared with the parental cDNAs in a multiple sequences alignment using ClustalX software.

eYACs construction

DNA preparation

As previously described, all cDNA molecules used in the eYACs preparation were cloned into a group of entry vectors between a yeast promoter and a yeast transcription terminator signal. 3 ml of the chimeric genes library previously assembled and 600 µl of the parental cDNAs library were separately inoculated from -80 °C glycerol stocks into two solutions of 250 ml LB medium with 100 µg/ml Ampicillin and incubated overnight at 37°C shaking at 180 rpm. Isolation of DNA plasmids was performed using the PureYield Plasmid MaxiPrep System following the manufacturer's protocols. The purified DNA obtained from different MaxiPrep procedures was ethanol precipitated and concentrated in a low final volume. The final concentration was verified using NanoDrop 2000C spectrophotometer.

Restriction digestion of the cDNA library

Entry vectors containing triterpenoid synthase parental and chimeric cDNAs of interest, *ARSH4*, and *URA3* auxotrophic marker were double digested with *NotI* and *Ascl*. The *S. cerevisiae* replication signal *ARSH4* and the *URA3* auxotrophic marker genes cloned in different entry vectors were provided by Evolva and after treatment with *Ascl* and *NotI* released the elements for replication and selection of the chromosomes in yeast. To limit the chances of spontaneous homologous recombination during eYAC construction a "non-functional" exogenous cDNA library, provided by Evolva and previously cloned in the same mix of entry vectors was digested and added to the preparation. A total amount of 50 µg DNA per preparation was used for different eYAC construction experiments (**Table 7.4**). All digestion reactions were carried out in a total volume 40 µl with 50 U of *NotI*, 50 U of *Ascl* and 1x Cutsmart Buffer. Reactions were incubated overnight at 37°C. The enzymes were then heat inactivated through incubation of the mixture at 65 °C for 20 min. In order to sequester away the short stopper fragment generated by *NotI* and *Ascl* digestion, 500 µM of competing oligonucleotide were added to every reaction and the temperature was set at 70°C for 20 min before to be cooled down at 25-20°C.

Table 7.4. DNA mixtures used in different eYACs preparation reactions.

	Reaction 1	Reaction 2	Reaction 3	Reaction 4	Reaction 5	Reaction 6	Reaction 7
ARSH4	2.0%	2.0%	2.0%	2.0%	2.0%	2.0%	2.0%
URA3 expression cassette	4.0%	4.0%	4.0%	4.0%	4.0%	4.0%	4.0%
Parental trit. synthase cDNAs	—	45.0%	—	—	—	15.0%	15.0%
Exogenous cDNA	94.0%	49.0%	69%	44.0%	19.0%	69.0%	44.0%
Chimeric trit. synthase cDNAs	—	—	25.0%	50.0%	75.0%	10.0%	35.0%

Concatenation reaction and eYAC arms ligation

In order to concatenate the expression cassettes, ATP at a final concentration of 5 mM, and 5.2 U of T4 ligase (Agilent) were added to each tube. Reactions were incubated 2 hours at room temperature. The ligase was then heat inactivated at 65 °C for 15 min. To check the degree of concatenation, the reactions were carefully pipetted using a 20 µl tips and the high molecular weight was detected by an increase in the viscosity of the DNA solution. eYAC arms containing *LEU2* auxotrophic marker gene have been prepared and digested in advance at Evolva. 40 µg of YAC arms, 1x Cutsmart Buffer, 1mM ATP, 5.6 units of T4 ligase (Agilent) in a total of 60 µl were added to every eYAC preparation reaction and gently mixed by pipetting. Reactions were incubated 30 min at room temperature, and the ligase was heat inactivated at 65 °C for 15 min.

Spheroplasts transformation

Two days before transformation, a single colony of EYS1019 yeast strain was inoculated into 50 ml of YPD broth at 30°C, shaking at 180 rpm for 24 hours. The day after 0.3 and 0.8 ml of the pre-culture were inoculated into 2 1000 ml YPD flasks and incubated overnight at 30 °C with shaking, till 3-5 OD₆₀₀. The next morning, OD₆₀₀ of the cultures was measured and 1000-1100 OD₆₀₀ units were harvested by centrifugation at 4000 rpm for 5 min. The supernatant was discarded and the cell pellet was washed twice, resuspending first in 50 ml of sterile nanopure water and then in 25 ml nanopure

water with 1M sorbitol. The solution was spun again at 4000 rpm for 5 min and the supernatant discarded before to resuspend the cells in 12.5 ml of SCE solution. The OD₆₀₀ was measured. 125 µl of 100 T Zymolyase were added, and the cells were incubated horizontally at 30 °C for 30 min with gentle shaking at 50-70 rpm, measuring every 5-10 min the OD₆₀₀, until it reached 20-30 % of the initial value. Once achieved the desired OD₆₀₀ value, the tubes were centrifuged at 1000 rpm at room temperature for 5 min, the supernatant was discarded, 20 ml of 1M sorbitol were added to each tube and the cells were gently mixed. The centrifugation and resuspension steps were repeated two more times resuspending before in 20 ml of STC and then in 6 ml of STC containing 50 µg/ml of calf-thymus DNA.

One falcon tube (15 ml) with 300 µl of spheroplasts was prepared for each transformation experiment and for positive and negative controls. Each eYAC preparation was added to the spheroplasts and the mixture gently mixed. As positive control 4 ng of plasmid carrying the same auxotrophic markers of the eYACs were used. As negative control, sterile water substituted the DNA preparation. 3 ml of PEG solution were then added to each tube and the samples incubated 5 min at room temperature before pelleting at 1000 rpm for 5 min. The supernatant was discarded and the cells resuspended in 1 ml of SOS and incubated at 30 °C for 40 min without shaking. For plating, top agar (2.5% noble agar and CSM-Leu), prewarmed at 45-50 °C, was used: 7 ml of molten top agar were mixed with the sample and the suspension was distributed onto SD (2% glucose) sorbitol transformation plates without leucine. The plates were incubated at 30 °C for 6 days, preventing drying. Transformants were restreaked as single colonies in SD (2% glucose) plates without uracil and leucine for further analysis.

Triterpenoids GC-MS detection

Cloning and expression of the triterpenoid synthase cDNAs

Four parental cDNAs codifying for β -amyrin synthases and 20 chimeric cDNAs were subcloned into pYES2.1 plasmid previously modified to accept *Hind*III and *Sac*II digested fragments between *GAL1* promoter and *CYC1* terminator. Transformation in EYS1019 yeast strain of all the plasmids was performed following a typical yeast lithium acetate transformation protocol (Gietz and Woods, 2002). Positive transformants were selected on agar plates without uracil to confirm the presence of the plasmid. Before to progress to GC-MS analyses each colony was inoculated for 24 hours at 30°C and 195 rpm in SD (2% glucose) liquid medium without uracil. Then part of the colony was washed with sterile water and inoculated in 5 ml of SD (2% galactose) without uracil at final OD₆₀₀ of 0.1 to trigger the cDNA expression under the control on *GAL1* promoter. The cultures were then kept at 30°C and shaken at 195 rpm for 24 hours. The day after, the samples were washed twice with sterile water and then centrifuged to collect the pellet. Positive EYS1019 colonies selected after eYAC transformation were grown on SD (2% Glucose) medium without leucine and uracil for 24 hour at 30°C. The day after the cultures were diluted at 0.1 OD₆₀₀ in the same medium and kept at 30°C for additional 72 hours. Finally the cultures were washed and collected prior to analyze the organic extracts through GC-MS.

Organic phase extraction

Each sample was resuspended in 1 ml of 60% ethanol solution and incubated at 78°C for 10 min with strong shacking. Afterwards samples were centrifuged at highest speed in a table top centrifuge and the supernatant was transferred to a 13 ml glass tube. Two volumes of hexane were added and the sample was vortexed thoroughly for 30 seconds. After a short spin the upper phase was transferred to glass vials and dried through EZ-2 Personal Evaporator according to manufacturer's instructions. All the samples were then resuspended in 50 μ l hexane and transferred in glass vials suitable for GC-MS injection.

GC-MS analysis

GC-MS analyses of the organic extracts were conducted using an Agilent 5973N mass spectrometer connected to a gas chromatograph 6890N (Agilent) with a DB-35ms (length: 30 m; diameter: 0.25 mm; film thickness: 0.25 μ m) capillary column. The injection temperature was 250°C and the column temperature program as follows: 1.5 min at 110°C followed by a rise to at a rate of 20°C/min 300°C. The final run time was 27 min. Helium with a flow rate of 1.2 ml/min was used as carrier gas. The interface temperature was 300°C and a splitless injection mode was used.

Yeast-based 11 β -HSD1 assays

Strains construction and growth conditions

All *Saccharomyces cerevisiae* strains constructed in this work (**Table 7.5**) derive from the S150-2B strain (MATa, *his3- Δ 1*, *leu2-3 112*, *trp1-289*, *ura3-52*). Yeast precultures were grown in YP medium plus 2% glucose (YPD) at 30°C. The integration plasmids carrying the construct *GRE-yEGFP* and *GRE-TEV* were provided by Evolva SA. The *TEV* and *yEGFP* genes are under the control of a *3xGRE+CYC1-TATA* promoter that efficiently activates downstream reporter constructs in yeast upon binding the glucocorticoid receptor (**Picard et al., 1990**). In RVY97 and RVY102 strains, the respective *GRE-GFP* and *GRE-TEV* constructs were integrated as a single copy into the ECM3 locus of chromosome XV of the yeast genome. Positive transformants were selected on YPD agar plates containing Hygromycin (200 μ g/ml). In the RVY102 assay strain, the N-Degron fluorescent reporter protein construct (Evolva SA), controlled by *GAL1* promoter was integrated as a single copy into the genome at the KIN1 locus of chromosome IV. The positive transformants were selected on YPD agar plates containing Nourseothricin (1 mg/ml). All integration processes were carried out using the lithium acetate transformation protocol (Gietz and Woods, 2002) and successful integration confirmed by PCR. Two plasmids carrying the murine *Nr3c1* cDNA and the wild type *HSD11B1* human cDNA codifying for GR and 11 β -HSD1 respectively were provided by Evolva SA. Both sequences were amplified by PCR and cloned via TA cloning in pYES 2.1 (Invitrogen) downstream of the *GAL1* promoter. The *HSD11B1* cDNA was cloned without stop codon and in frame with a sequence coding for a polyhistidine tag. The *GAL1- HSD11B1* expression cassette was then amplified by PCR from the pYES 2.1 vector and sub-cloned into a plasmid with a *TRP1* auxotrophic marker (pEVE2113) whereas *Nr3c1* was kept in the pYES 2.1 (*URA3*). The transformation of both ectopic plasmids carrying the *Nr3c1* and *HSD11B1*

cDNAs was performed using the lithium acetate transformation protocol. Positive clones were selected on SD agar plates (2% glucose) lacking uracil and tryptophan for double plasmid selection. All the genetic constructs used for strain construction were confirmed by sequencing.

All experiments with strains RVY96 or RVY97 and RVY101 or RVY102 were carried out in SD medium (2% glucose) without uracil and tryptophan for 24 hours and then washed and re-suspended in SD medium (2% galactose) without uracil and tryptophan for induction of GR and 11 β -HSD1 expression. Cortisone and dexamethasone were added to the cultures from 28 mM stock solutions in DMSO to the required concentrations.

Table 7.5. Genotypes of the yeast strains assembled in this work.

Strain	Genotype
RVY96	<i>S1502B-ECM3::pCYC 3xGRE-yEGFP-tADH1 loxP-HygR-loxP [Nr3c1-TRP1-2μ]</i>
RVY97	<i>S1502B-ECM3::pCYC 3xGRE-yEGFP-tADH1 loxP-HygR-loxP [Nr3c1-TRP1-2μ] [HSD11B1-URA3-2μ]</i>
RVY101	<i>S1502B-ECM3::pCYC3xGRE-TEV-tADH1 loxP-HygR-loxP KIN1::GAL1-Degron-tADH1 loxP-NatR-loxP [Nr3c1-TRP1-2μ]</i>
RVY102	<i>S1502B-ECM3::pCYC3xGRE-TEV-tADH1 loxP-HygR-loxP KIN1::GAL1-Degron-tADH1 loxP-NatR-loxP [Nr3c1-TRP1-2μ] [HSD11B1-URA3-2μ]</i>

Validation of the assays with carbenoxolone

Yeast strains were maintained for 24 hours in 2% glucose SD medium without uracil and tryptophan. After 24 hours, the cells were diluted to an OD₆₀₀ of 0.1 in 2% galactose SD medium lacking uracil and tryptophan. The cultures were grown for an additional 18 hours in galactose to induce 11 β -HSD1, GR and the N-Degron fluorescent fusion protein expression and then divided into 3 ml subcultures. Cortisone was added to the cultures from a 28 mM stock solution in DMSO to the required concentrations. At different time points the cells were collected, resuspended in 1x PBS and analysed using a FACSCalibur flow cytometer from Becton Dickson (San Jose, CA). Every experiment was repeated independently at least three times.

The specificity of the signal upon treatment with cortisone was confirmed with strains in which the plasmids carrying the 11 β -HSD1 expression cassette were replaced by the equivalent empty plasmids. Except for the missing 11 β -HSD1 expression construct, the strains were identical to the two

parental assay strains RVY97 and RVY102. In the validation of the assays the 11 β -HSD1 inhibitor carbenoxolone (CBX) (stock solution 1 mM/water) was added to 10⁶ cells together with cortisone to reach a final concentration of 0.1, 0.3, 1.0 and 3.0 μ M, respectively. Cells were incubated for 6 hours and after washing in 1x PBS the samples were analysed by FACS. Both RVY97 and RVY102 strains were tested with carbenoxolone under identical conditions.

Figure 4.19a shows the green fluorescence of RVY102 samples treated with 1000 μ M cortisone and increasing concentration of carbenoxolone (CBX). The percentage of γ EGFP degradation events obtained upon treatment of RVY102 cells with 1000 μ M cortisone and no carbenoxolone was set at 1. This value was used as reference for the normalization of the other degradation values. In order to get the fluorescence values plotted, the formula (fluorescence = 1- degradation value) was used.

Flow cytometric and fluorescence microscopy analysis of yeast fluorescence

250 μ l of yeast culture volume were centrifuged and re-suspended in 500 μ l 1x PBS. FACS analyses were performed using the FACSCalibur instrument. The instrument settings were as follows: log forward scatter (FSC) E00; log side scatter 2 (SSC) at 458 V, log FL1 fluorescence at 468 V and log FL2 fluorescence at 460 V. The γ EGFP fluorescence was excited at 488 nm and collected through 530/30 nm band-pass filter on the FL1 channel. The tdTomato fluorescence was excited at 488 nm and collected through 585/42 nm band-pass filter on the FL2 channel. For multicolour analysis of the strain RVY102, fluorescence compensation (FL2- 20.8% FL1) was manually set using single colour cell controls. The typical sampling rate was 200 events/s, and the typical sample size was 10'000 cells per measurement. The data were analysed with FlowJo vX.0.7 software. For fluorescence images the samples were taken at different dilutions and examined for green and red fluorescence under the optical microscope BX41 (Olympus) equipped with FITC and TRITC filter sets using a 40x magnification lens and an exposure time of 1/3 sec.

Rhodamine 6G accumulation and efflux

The strains RVY97 and RVY102 (OD₆₀₀ of 0.1) were incubated in the presence of FK506 (10 μ M) or DMSO (control) for 20 min followed by incubation with 5 μ M Rhodamine 6G for 1 hour at 30°C. 10'000 cells of each population were analysed by FACS.

Sensitivity to cortisone in presence of FK506

The 11 β -HSD1 assay strains RVY97 and RVY102 were tested for the cortisone induced gene transactivation after incubation with 10 μ M FK506 or DMSO for 20 min at 30°C.

8

Materials

Cells

EYS1019 (yeast strain)	(Kindly provided by Evolva)
NEB 5-alpha Competent <i>E. coli</i>	(NEB, Cat. No C2988J)
NEB 5-alpha Competent <i>E. coli</i> (High Efficiency)	(NEB, Cat. No C29871)
RVY101 (yeast strain)	(Constructed in this work)
RVY102 (yeast strain)	(Constructed in this work)
RVY96 (yeast strain)	(Constructed in this work)
RVY97 (yeast strain)	(Constructed in this work)
S150-2B (yeast strain)	(Kindly provided by Prof. Dr. Georg Lipps)

Table 8.1. Genotypes of the bacterial and yeast strains used in this study.

Strain	Genotype
NEB 5-alpha Competent <i>E. coli</i> (Subcloning Efficiency)	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>
NEB 5-alpha Competent <i>E. coli</i> (High Efficiency)	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>
EYS1019	<i>MATalpha his3Δ0 leu2Δ0 lys2Δ0 trp1Δ0 ura3Δ0 arg4Δ0</i>
S150-2B	<i>MATa, his3-Δ1, leu2-3 112, trp1-289, ura3-52</i>
RVY96	<i>S1502B-ECM3::pCYC 3xGRE-yEGFP-tADH1 loxP-HygR-loxP [Nr3c1-TRP1-2μ]</i>
RVY97	<i>S1502B-ECM3::pCYC 3xGRE-yEGFP-tADH1 loxP-HygR-loxP [Nr3c1-TRP1-2μ] [HSD11B1-URA3-2μ]</i>
RVY101	<i>S1502B-ECM3::pCYC3xGRE-TEV-tADH1 loxP-HygR-loxP KIN1::GAL1 Degron-tADH1 loxP-NatR-loxP [Nr3c1-TRP1-2μ]</i>
RVY102	<i>S1502B-ECM3::pCYC3xGRE-TEV-tADH1 loxP-HygR-loxP KIN1::GAL1 Degron-tADH1 loxP-NatR-loxP [Nr3c1-TRP1-2μ] [HSD11B1-URA3-2μ]</i>

Enzymes and DNA ladders

1kb plus DNA Ladder	(Invitrogen, Cat. No. 15615-016)
Ascl	(NEB, Cat. No. R0558S)
FIREPol DNA Polymerase	(Solis BioDyne, Cat. No. 01-01-00500)
<i>Hind</i> III-HF	(NEB, Cat. No. R3104M)
<i>Not</i> I-HF	(NEB, Cat. No. R3189L)
Phusion High-Fidelity DNA Polymerase	(NEB, Cat. No. M0530L)
Quick-Load 1 kp DNA Ladder	(NEB, Cat. No. N0468S)
Quick-Load 50 bp DNA Ladder	(NEB, Cat. No. N0473S)
<i>Sac</i> II	(NEB, Cat. No. R0157L)
T4 DNA Ligase	(NEB, Cat. No. M0202S)
T4 DNA Ligase	(Agilent, Cat. No. 600011)
Zymolyase 100T	(Zymo Reasearch, Cat. No. E1005)

Products and reagents

10x Buffer B	(Solis BioDyne, Cat. No. 01-01-00500)
10X Cutsmart Buffer	(NEB, Cat. No. B7204S)
10X T4 DNA Ligase Buffer (with ATP)	(NEB, Cat. No. B0202S)
5x Phusion HF reaction Buffer	(NEB, Cat. No. B0518S)
Adenine	(Sigma-Aldrich, Cat. No. A8626)
Agar	(Sigma-Aldrich, Cat. No. A1296)
Ampicillin	(Carl Roth, Cat. No K029.1)
ATP	(Fermentas Cat. No. R0441)
CaCl ₂	(Sigma-Aldrich, Cat. No. 223506)
Calf thymus DNA	(Sigma-Aldrich, Cat. No. D4522)
Carbenoxolone	(Sigma-Aldrich, Cat. No. C4790)
Cortisone	(Sigma-Aldrich, Cat. No. C2755)
CSM-Leu	(MPBio, Cat. No. 114510512)
CSM-Leu-Ura	(MPBio, Cat. No. 114520212)
D(+)-Galactose	(Roth, Cat. No. 4987.2)

Dexamethasone	(Sigma-Aldrich, Cat. No. D4902)
Dimethyl Sulfoxide	(Thermo Scientific, Cat. No. 20688)
dNTPs Mix	(Solis BioDyne, Cat. No. 02-31-00020)
EDTA	(Sigma-Aldrich, Cat. No. 1233508)
Ethanol	(Sigma-Aldrich, Cat. No. 02854)
Ethidium Bromide	(Sigma-Aldrich, Cat. No. E1510)
FK-506 monohydrate	(Sigma-Aldrich, Cat. No. F4679)
Gel Loading Dye Purple (6X)	(NEB, Cat. No. B70245)
Glycerol	(Sigma-Aldrich, Cat. No. G5516)
Hexane	(Sigma-Aldrich, Cat. No. 34859)
Hydrocortisone	(Sigma-Aldrich, Cat. No. H0888)
Hygromycin B (Hygr)	(Sigma-Aldrich, Cat. No. H3274)
LB Broth with agar	(Sigma-Aldrich, Cat. No. L2897)
L-Histidine	(Sigma-Aldrich, Cat. No. 53320)
Lithium acetate dihydrate	(Sigma-Aldrich, Cat. No. L4158)
L-Leucine	(Sigma-Aldrich, Cat. No. 61819)
L-Lysine	(Sigma-Aldrich, Cat. No. L5501)
L-Methionine	(Sigma-Aldrich, Cat. No. M9625)
L-Tryptophan	(Sigma-Aldrich, Cat. No. 93659)
Luria Broth	(Sigma-Aldrich, Cat. No. L3522)
MgCl ₂	(Solis BioDyne, Cat. No. 01-01-00500)
Noble Agar	(Difco, Cat. No. 214220)
Nourseothricin (Nat)	(Sigma-Aldrich, Cat. No. 74667)
Peptone from meat and soybean meal	(Sigma-Aldrich, Cat. No. 93733)
Polyethylene glycol 4000	(Sigma-Aldrich, Cat. No. 5904)
Rhodamine 6G	(Sigma-Aldrich, Cat. No. R4127)
Salmon Sperm DNA	(Invitrogen, Cat. No. 15632-011)
Sodium Citrate	(Sigma-Aldrich, Cat. No. W302600)
Sorbitol	(Sigma-Aldrich, Cat. No. S1876)
Tris base	(Sigma-Aldrich, Cat. No. 252859)
Ultra Pure TM Agarose	(Invitrogen, Cat. No. 15510-027)

Uracil	(Sigma-Aldrich, Cat. No. 94220)
Yeast Extract	(Sigma-Aldrich, Cat. No. 92144)
Yeast Nitrogen Base without Amino Acids	(Sigma-Aldrich, Cat. No. Y0626)
Yeast Synthetic Drop-out Medium Supplements without histidine, leucine, tryptophan and uracil	(Sigma-Aldrich, Cat. No. Y2001)
α -D-Glucose	(Sigma-Aldrich, Cat. No. 158968)

Plasmids

entry vectors (Table 7.3)	(Kindly provided by Evolva)
pET303/CT-His	(Life Technologies, Cat. No.K630001)
pEVE1801	(Kindly provided by Evolva)
pEVE1807 (<i>GAL1</i> promoter)	(Kindly provided by Evolva)
pEVE1889	(Kindly provided by Evolva)
pEVE2113	(Kindly provided by Evolva)
pYES2.1/V5-His-TOPO	(Life Technologies, Cat. No. K4150-01)

Kits

PCR clean-up & Gel extraction: NucleoSpin Extract II	(Macherey-Nagel, Cat. No. 740 609)
PureYield Plasmid Miniprep System	(Promega, Cat. No. A1223)
PureYield Plasmid Maxiprep System	(Promega, Cat. No. A2393)
pYES2.1 TOPO TA Expression Kit	(Life Technologies, Cat. No. K4150-01)

Instruments

BX41, System Microscope	(Olympus)
Centrifuge 5418 R	Eppendorf,5418.0000.017)
Centrifuge 5804 R	(Eppendorf,5805000.327)
EZ-2 Personal Evaporator	(Genevac)
FACS Calibur	(Becton Dickinson)
Gas Chromatograph 6890N	(Agilent)
Mass Spectrometer 5973N	(Agilent)

Multimage TM Light Cabinet	(Alpha Innotech)
Nanodrop 2000c	(Thermo Scientific)
peqSTAR 2X Gradient Thermocycler	(peqlab, 95-08002)
T3000 Thermocycler	(Biometra)
Thermomixer Comfort	(Eppendorf, 5355000.011)
U-RFL-T, Power Supply Unit	(Olympus)

Software and algorithms

Basic Local Alignment Search Tool (BLAST)	(www.ncbi.nlm.nih.gov)
BD CellQuest Pro Software	(Becton Dickinson)
Sequence Scanner v1.0	(Applied Biosystems)
FluroChem™ SP Alpha Innotech	(Version 4.1.0)
Compass data analysis 4.0	(Bruker)
FlowJo vX.0.7.	(http://docs.flowjo.com/d2/)
NanoDrop 2000/2000C	(Version 1.4.1)
Snap Gene® Viewer	(Version 1.5.3)
ClustalX	(Version 2.1)

Growth media preparation

YPD: Yeast extract 10g/L , Peptone 20g/L, α -D-Glucose 20g/L

SD w/o uracil (2% Glucose): Yeast nitrogen base without amino acids 6.7 g/L, Yeast synthetic drop-out media supplement without uracil 1.92 g/L, α -D-Glucose 20 g/L

SD w/o uracil (2% Galactose): Yeast nitrogen base without amino acids 6.7 g/L, Yeast synthetic drop-out media supplement without uracil 1.92 g/L, D(+)-Galactose 20g/L

SD w/o uracil and tryptophan (2% Glucose): Yeast nitrogen base without amino acids 6.7 g/L, Yeast synthetic drop-out media supplement without uracil tryptophan histidine and leucine 1.39 g/L, leucine 380 mg/L, histidine 76 mg/L, α -D-Glucose 20 g/L

SD w/o uracil and tryptophan (2% Galactose): Yeast nitrogen base without amino acids 6.7 g/L, Yeast synthetic drop-out media supplement without uracil tryptophan histidine and leucine 1.39 g/L, leucine 380 mg/L, histidine 76 mg/L, D(+)-Galactose 20g/L

For agar plates production the equivalent media were completed with 20 g/L agar before sterilization. In plates preparation Glucose and Galactose were added at 2% (w/v) final concentration from a sterile solution of 20% (w/v) just after autoclaving.

Eventual antibiotics were added to yeast agar plates after autoclaving at the final working concentration (Nat, 60 µg/ml and Hygr, 200 µg/ml)

Solutions

SCE: (1M Sorbitol, 0.1M Sodium Citrate pH 7.6, 0.06M EDTA)

STC: (0.98 M Sorbitol, 10 mM Tris pH 7.5, 10 mM CaCl₂)

PEG8000: (19.6% PEG 8000 w/v, 10 mM tris pH 7.5, 10 mM CaCl₂)

SOS: (2 M sorbitol, 7 mM CaCl₂, 25 % v/v YPD medium) + Amino acid mix (URA, HIS, LEU, TRP, ADE, LYS, MET 20 mg/l each) + Water

PBS (10x): (80.6mM sodium phosphate, 19.4mM potassium phosphate, 27mM KCl and 1.37M NaCl, pH 7.4)

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Bibliography

- Agarwal MK, Mirshahi M. **General overview of mineralocorticoid hormone action.** Pharmacol Ther. 1999 Dec;84(3):273-326.
- Albiston AL, Obeyesekere VR, Smith RE, Krozowski ZS. **Cloning and tissue distribution of the human 11 beta-hydroxysteroid dehydrogenase type 2 enzyme.** Mol Cell Endocrinol. 1994 Nov;105(2):R11-7.
- Anbalagan M, Huderson B, Murphy L, Rowan BG. **Post-translational modifications of nuclear receptors and human disease.** Nucl Recept Signal. 2012;10:e001.
- Andersen CY. **Possible new mechanism of cortisol action in female reproductive organs: physiological implications of the free hormone hypothesis.** J Endocrinol. 2002 May;173(2):211-7.
- Arampatzis S, Kadereit B, Schuster D, Balazs Z, Schweizer RA, Frey FJ, Langer T, Odermatt A. **Comparative enzymology of 11beta-hydroxysteroid dehydrogenase type 1 from six species.** J Mol Endocrinol. 2005 Aug;35(1):89-101.
- Atanasov AG, Dzyakanchuk AA, Schweizer RA, Nashev LG, Maurer EM, Odermatt A. **Coffee inhibits the reactivation of glucocorticoids by 11beta-hydroxysteroid dehydrogenase type 1: a glucocorticoid connection in the anti-diabetic action of coffee?** FEBS Lett. 2006 Jul 24;580(17):4081-5.
- Atanasov AG, Nashev LG, Gelman L, Legeza B, Sack R, Portmann R, Odermatt A. **Direct protein-protein interaction of 11beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase in the endoplasmic reticulum lumen.** Biochim Biophys Acta. 2008 Aug;1783(8):1536-43.
- Atanasov AG, Nashev LG, Schweizer RA, Frick C, Odermatt A. **Hexose-6-phosphate dehydrogenase determines the reaction direction of 11beta-hydroxysteroid dehydrogenase type 1 as an oxoreductase.** FEBS Lett. 2004 Jul 30;571(1-3):129-33.
- Atanasov AG, Odermatt A. **Readjusting the glucocorticoid balance: an opportunity for modulators of 11beta-hydroxysteroid dehydrogenase type 1 activity?** Endocr Metab Immune Disord Drug Targets. 2007 Jun;7(2):125-40.
- Augustin JM, Kuzina V, Andersen SB, Bak S. **Molecular activities, biosynthesis and evolution of triterpenoid saponins.** Phytochemistry. 2011 Apr;72(6):435-57.
- Bachmair A, Finley D, Varshavsky A. **In vivo half-life of a protein is a function of its amino-terminal residue.** Science. 1986 Oct 10;234(4773):179-86.
- Baker ME. **Unusual evolution of 11beta- and 17beta-hydroxysteroid and retinol dehydrogenases.** Bioessays. 1996 Jan;18(1):63-70.
- Barberis A, Gunde T, Berset C, Audetat S, Lüthi U. **Yeast as a screening tool.** Drug Discov Today Technol. 2005 Summer;2(2):187-92.
- Barf T, Vallgård J, Emond R, Häggström C, Kurz G, Nygren A, Larwood V, Mosialou E, Axelsson K, Olsson R, Engblom L, Edling N, Rönquist-Nii Y, Ohman B, Alberts P, Abrahmsén L. **Arylsulfonamidothiazoles as a new class of potential antidiabetic drugs. Discovery of potent and selective inhibitors of the 11beta-hydroxysteroid dehydrogenase type 1.** J Med Chem. 2002 Aug 29;45(18):3813-5.
- Barton PJ, Clarke DS, Davies CD, Hargreaves RB, Pease JE, Rankine MT. World patent. 2004; WO2004/011410 (A1), Chemical Compounds.

- Baxter JD. **Glucocorticoid hormone action**. *Pharmacol Ther B*. 1976;2(3):605-69.
- Berliner, D. L. & Dougherty, T. F. **Hepatic and extrahepatic regulation of corticosteroids**, *Pharmacol. Rev.* 1961 Sep 13, 329 -359.
- Beseda I, Czollner L, Shah PS, Khunt R, Gaware R, Kosma P, Stanetty C, Del Ruiz-Ruiz MC, Amer H, Mereiter K, Da Cunha T, Odermatt A, Classen-Houben D, Jordis U. **Synthesis of glycyrrhetic acid derivatives for the treatment of metabolic diseases**. *Bioorg Med Chem*. 2010 Jan 1;18(1):433-54.
- Biddie SC, Conway-Campbell BL, Lightman SL. **Dynamic regulation of glucocorticoid signalling in health and disease**. *Rheumatology (Oxford)*. 2012 Mar;51(3):403-12
- Blum A, Favia AD, Maser E. **11beta-Hydroxysteroid dehydrogenase type 1 inhibitors with oleanan and ursan scaffolds**. *Mol Cell Endocrinol*. 2009 Mar 25;301(1-2):132-6.
- Bujalska IJ, Draper N, Michailidou Z, Tomlinson JW, White PC, Chapman KE, Walker EA, Stewart PM. **Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11 beta-hydroxysteroid dehydrogenase type 1**. *J Mol Endocrinol*. 2005 Jun;34(3):675-84.
- Castro A, Zhu JX, Alton GR, Rejto P, Ermolieff J. **Assay optimization and kinetic profile of the human and the rabbit isoforms of 11beta-HSD1**. *Biochem Biophys Res Commun*. 2007 Jun 1;357(2):561-6. Epub 2007 Apr 9. PubMed PMID: 17434447.
- Chapman K, Holmes M, Seckl J. **11 β -hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action**. *Physiol Rev*. 2013 Jul;93(3):1139-206.
- Coleman JJ, Okoli I, Tegos GP, Holson EB, Wagner FF, Hamblin MR, Mylonakis E. **Characterization of plant-derived saponin natural products against *Candida albicans***. *ACS Chem Biol*. 2010 Mar 19;5(3):321-32.
- Cope CL, Black E. **The production rate of cortisol in man**. *Br Med J*. 1958 May 3;1(5078):1020-4.
- Corey EJ, Matsuda SP, Bartel B. **Isolation of an *Arabidopsis thaliana* gene encoding cycloartenol synthase by functional expression in a yeast mutant lacking lanosterol synthase by the use of a chromatographic screen**. *Proc Natl Acad Sci U S A*. 1993 Dec 15;90(24):11628-32.
- Corey EJ, Matsuda SP, Bartel B. **Molecular cloning, characterization, and overexpression of ERG7, the *Saccharomyces cerevisiae* gene encoding lanosterol synthase**. *Proc Natl Acad Sci U S A*. 1994 Mar 15;91(6):2211-5.
- Crameri A, Raillard SA, Bermudez E, Stemmer WP. **DNA shuffling of a family of genes from diverse species accelerates directed evolution**. *Nature*. 1998 Jan 15;391(6664):288-91.
- Day C. **Metabolic syndrome, or What you will: definitions and epidemiology**. *Diab Vasc Dis Res*. 2007 Mar;4(1):32-8.
- Desbriere R, Vuaroqueaux V, Achard V, Boullu-Ciocca S, Labuhn M, Dutour A, Grino M. **11beta-hydroxysteroid dehydrogenase type 1 mRNA is increased in both visceral and subcutaneous adipose tissue of obese patients**. *Obesity (Silver Spring)*. 2006 May;14(5):794-8.
- Duperrex H, Kenouch S, Gaeggeler HP, Seckl JR, Edwards CR, Farman N, Rossier BC. **Rat liver 11 beta-hydroxysteroid dehydrogenase complementary deoxyribonucleic acid encodes oxoreductase activity in a mineralocorticoid-responsive toad bladder cell line**. *Endocrinology*. 1993 Feb;132(2):612-9.

- Escher G, Vogt B, Beck T, Guntern D, Frey BM, Frey FJ. Reduced 11beta-hydroxysteroid dehydrogenase activity in the remaining kidney following nephrectomy. *Endocrinology*. 1998 Apr;139(4):1533-9
- Esteban NV, Loughlin T, Yergey AL, Zawadzki JK, Booth JD, Winterer JC, Loriaux DL. **Daily cortisol production rate in man determined by stable isotope dilution/mass spectrometry.** *J Clin Endocrinol Metab*. 1991 Jan;72(1):39-45.
- Filling C, Berndt KD, Benach J, Knapp S, Prozorovski T, Nordling E, Ladenstein R, Jörnvall H, Oppermann U. **Critical residues for structure and catalysis in short-chain dehydrogenases/reductases.** *J Biol Chem*. 2002 Jul 12;277(28):25677-84.
- Fotsch C, Wang M. **Blockade of glucocorticoid excess at the tissue level: inhibitors of 11beta-hydroxysteroid dehydrogenase type 1 as a therapy for type 2 diabetes.** *J Med Chem*. 2008 Aug 28;51(16):4851-7.
- Frick C, Atanasov AG, Arnold P, Ozols J, Odermatt A. **Appropriate function of 11beta-hydroxysteroid dehydrogenase type 1 in the endoplasmic reticulum lumen is dependent on its N-terminal region sharing similar topological determinants with 50-kDa esterase.** *J Biol Chem*. 2004 Jul 23;279(30):31131-8
- Funder JW. **Mineralocorticoid receptors and glucocorticoid receptors.** *Clin Endocrinol Oxf*. 1996 Dec;45(6):651-6.
- Garabedian MJ, Yamamoto KR. **Genetic dissection of the signaling domain of a mammalian steroid receptor in yeast.** *Mol Biol Cell*. 1992 Nov;3(11):1245-57.
- Gibbs MD, Nevalainen KM, Bergquist PL. **Degenerate oligonucleotide gene shuffling (DOGS): a method for enhancing the frequency of recombination with family shuffling.** *Gene*. 2001 Jun 13;271(1):13-20.
- Gietz RD, Woods RA. **Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method.** *Methods Enzymol*. 2002;350:87-96.
- Gummy C, Thurnbichler C, Aubry EM, Balazs Z, Pfisterer P, Baumgartner L, Stuppner H, Odermatt A, Rollinger JM. **Inhibition of 11beta-hydroxysteroid dehydrogenase type 1 by plant extracts used as traditional antidiabetic medicines.** *Fitoterapia*. 2009 Apr;80(3):200-5.
- Hamberger B, Bak S. **Plant P450s as versatile drivers for evolution of species-specific chemical diversity.** *Philos Trans R Soc Lond B Biol Sci*. 2013 Jan 6;368(1612):20120426.
- Hayashi H, Huang P, Inoue K. **Up-regulation of soyasaponin biosynthesis by methyl jasmonate in cultured cells of *Glycyrrhiza glabra*.** *Plant Cell Physiol*. 2003 Apr;44(4):404-11.
- Hayashi H, Huang P, Kirakosyan A, Inoue K, Hiraoka N, Ikeshiro Y, Kushiro T, Shibuya M, Ebizuka Y. **Cloning and characterization of a cDNA encoding beta-amyrin synthase involved in glycyrrhizin and soyasaponin biosyntheses in licorice.** *Biol Pharm Bull*. 2001 Aug;24(8):912-6.
- Hermanowski-Vosatka A, Balkovec JM, Cheng K, Chen HY, Hernandez M, Koo GC, Le Grand CB, Li Z, Metzger JM, Mundt SS, Noonan H, Nunes CN, Olson SH, Pikounis B, Ren N, Robertson N, Schaeffer JM, Shah K, Springer MS, Strack AM, Strowski M, Wu K, Wu T, Xiao J, Zhang BB, Wright SD, Thieringer R. **11beta-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice.** *J Exp Med*. 2005 Aug 15;202(4):517-27.

- Herrera JB, Bartel B, Wilson WK, Matsuda SP. **Cloning and characterization of the Arabidopsis thaliana lupeol synthase gene.** Phytochemistry. 1998 Dec;49(7):1905-11.
- Hosfield DJ, Wu Y, Skene RJ, Hilgers M, Jennings A, Snell GP, Aertgeerts K. **Conformational flexibility in crystal structures of human 11beta-hydroxysteroid dehydrogenase type I provide insights into glucocorticoid interconversion and enzyme regulation.** J Biol Chem. 2005 Feb 11;280(6):4639-48.
- Iturbe-Ormaetxe I, Haralampidis K, Papadopoulou K, Osbourn AE. **Molecular cloning and characterization of triterpene synthases from Medicago truncatula and Lotus japonicus.** Plant Mol Biol. 2003 Mar;51(5):731-43.
- Kirby J, Keasling JD. **Biosynthesis of plant isoprenoids: perspectives for microbial engineering.** Annu Rev Plant Biol. 2009;60:335-55.
- Klein J, Heal JR, Hamilton WD, Boussemghoune T, Tange TØ, Delegrange F, Jaeschke G, Hatsch A, Heim J. **Yeast synthetic biology platform generates novel chemical structures as scaffolds for drug discovery.** ACS Synth Biol. 2014 May 16;3(5):314-23.
- Kolaczowski M, van der Rest M, Cybularz-Kolaczowska A, Soumillion JP, Konings WN, Goffeau A. **Anticancer drugs, ionophoric peptides, and steroids as substrates of the yeast multidrug transporter Pdr5p.** J Biol Chem.
- Kotelevtsev Y, Brown RW, Fleming S, Kenyon C, Edwards CR, Seckl JR, Mullins JJ. **Hypertension in mice lacking 11beta-hydroxysteroid dehydrogenase type 2.** J Clin Invest. 1999 Mar;103(5):683-9.
- Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, Best R, Brown R, Edwards CR, Seckl JR, Mullins JJ. **11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress.** Proc Natl Acad Sci U S A. 1997 Dec 23;94(26):14924-9.
- Kralli A, Bohlen SP, Yamamoto KR. **LEM1, an ATP-binding-cassette transporter, selectively modulates the biological potency of steroid hormones.** Proc Natl Acad Sci U S A. 1995 May 9;92(10):4701-5.
- Kralli A, Yamamoto KR. **An FK506-sensitive transporter selectively decreases intracellular levels and potency of steroid hormones.** J Biol Chem. 1996 Jul 19;271(29):17152-6.
- Kushiro T, Shibuya M, Ebizuka Y. **Beta-amyrin synthase--cloning of oxidosqualene cyclase that catalyzes the formation of the most popular triterpene among higher plants.** Eur J Biochem. 1998 Aug 15;256(1):238-44.
- Lakshmi V, Monder C. **Purification and characterization of the corticosteroid 11 beta-dehydrogenase component of the rat liver 11 beta-hydroxysteroid dehydrogenase complex.** Endocrinology. 1988 Nov;123(5):2390-8.
- Lavery GG, Walker EA, Draper N, Jeyasuria P, Marcos J, Shackleton CH, Parker KL, White PC, Stewart PM. **Hexose-6-phosphate dehydrogenase knock-out mice lack 11 beta-hydroxysteroid dehydrogenase type 1-mediated glucocorticoid generation.** J Biol Chem. 2006 Mar 10;281(10):6546-51.
- Lee YS, Lorenzo BJ, Koufis T, Reidenberg MM. **Grapefruit juice and its flavonoids inhibit 11 beta-hydroxysteroid dehydrogenase.** Clin Pharmacol Ther. 1996 Jan;59(1):62-71.
- Levesque J, Lamarche B. **The metabolic syndrome: definitions, prevalence and management.** J Nutrigenet Nutrigenomics. 2008;1(3):100-8.

- Lodeiro S, Schulz-Gasch T, Matsuda SP. **Enzyme redesign: two mutations cooperate to convert cycloartenol synthase into an accurate lanosterol synthase.** J Am Chem Soc. 2005 Oct 19;127(41):14132-3.
- Low SC, Chapman KE, Edwards CR, Seckl JR. **'Liver-type' 11 beta-hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian COS-7 cells.** J Mol Endocrinol. 1994 Oct;13(2):167-74.
- Mahé Y, Lemoine Y, Kuchler K. **The ATP binding cassette transporters Pdr5 and Snq2 of Saccharomyces cerevisiae can mediate transport of steroids in vivo.** J Biol Chem. 1996 Oct 11;271(41):25167-72.
- Man S, Gao W, Zhang Y, Huang L, Liu C. **Chemical study and medical application of saponins as anti-cancer agents.** Fitoterapia. 2010 Oct;81(7):703-14.
- Manday Viswanathan P. **Establishment of an in vitro assay for qualitative analysis of the activity of 11 β -HSD1.** University of Applied Sciences and Arts Northwestern Switzerland. School of Life Sciences, 2014 Oct.
- Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR, Flier JS. **A transgenic model of visceral obesity and the metabolic syndrome.** Science. 2001 Dec 7;294(5549):2166-70.
- Meulenber EP, Hofman JA. **The effect of pretreatment of saliva on steroid hormone concentrations.** J Clin Chem Clin Biochem. 1990 Dec;28(12):923-8.
- Meyer MM, Xu R, Matsuda SP. **Directed evolution to generate cycloartenol synthase mutants that produce lanosterol.** Org Lett. 2002 Apr 18;4(8):1395-8.
- Monder C, Stewart PM, Lakshmi V, Valentino R, Burt D, Edwards CR. **Licorice inhibits corticosteroid 11 beta-dehydrogenase of rat kidney and liver: in vivo and in vitro studies.** Endocrinology. 1989 Aug;125(2):1046-53.
- Morita M, Shibuya M, Kushiro T, Masuda K, Ebizuka Y. **Molecular cloning and functional expression of triterpene synthases from pea (Pisum sativum) new alpha-amyrin-producing enzyme is a multifunctional triterpene synthase.** Eur J Biochem. 2000 Jun;267(12):3453-60.
- Morris DJ, Souness GW, Latif SA, Hardy MP, Brem AS. **Effect of chenodeoxycholic acid on 11beta-hydroxysteroid dehydrogenase in various target tissues.** Metabolism. 2004 Jun;53(6):811-6.
- Morton NM, Holmes MC, Fiévet C, Staels B, Tailleux A, Mullins JJ, Seckl JR. **Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11beta-hydroxysteroid dehydrogenase type 1 null mice.** J Biol Chem. 2001 Nov 2;276(44):41293-300.
- Morton NM, Paterson JM, Masuzaki H, Holmes MC, Staels B, Fievet C, Walker BR, Flier JS, Mullins JJ, Seckl JR. **Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice.** Diabetes. 2004 Apr;53(4):931-8.
- Mune T, Rogerson FM, Nikkilä H, Agarwal AK, White PC. **Human hypertension caused by mutations in the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase.** Nat Genet. 1995 Aug;10(4):394-9
- Naesby M, Nielsen SV, Nielsen CA, Green T, Tange TO, Simón E, Knechtle P, Hansson A, Schwab MS, Titiz O, Folly C, Archila RE, Maver M, van Sint Fiet S, Boussemerghoune T, Janes M, Kumar AS, Sonkar SP, Mitra PP, Benjamin VA, Korrapati N, Suman I, Hansen EH, Thybo T, Goldsmith N, Sorensen AS. **Yeast artificial chromosomes employed for random assembly of biosynthetic pathways and production of diverse compounds in Saccharomyces cerevisiae.** Microb Cell Fact. 2009 Aug 13;8:45.

- Náray-Fejes-Tóth A, Fejes-Tóth G. **Subcellular localization of the type 2 11beta-hydroxysteroid dehydrogenase. A green fluorescent protein study.** J Biol Chem. 1996 Jun 28;271(26):15436-42.
- Ness JE, Welch M, Giver L, Bueno M, Cherry JR, Borchert TV, Stemmer WP, Minshull J. **DNA shuffling of subgenomic sequences of subtilisin.** Nat Biotechnol. 1999 Sep;17(9):893-6.
- Nordling E, Jörnvall H, Persson B. **Medium-chain dehydrogenases/reductases (MDR). Family characterizations including genome comparisons and active site modeling.** Eur J Biochem. 2002 Sep;269(17):4267-76.
- Oakley RH, Cidlowski JA. **Cellular processing of the glucocorticoid receptor gene and protein: new mechanisms for generating tissue-specific actions of glucocorticoids.** J Biol Chem. 2011 Feb 4;286(5):3177-84.
- Odermatt A, Arnold P, Stauffer A, Frey BM, Frey FJ. **The N-terminal anchor sequences of 11beta-hydroxysteroid dehydrogenases determine their orientation in the endoplasmic reticulum membrane.** J Biol Chem. 1999 Oct 1;274(40):28762-70.
- Odermatt A, Atanasov AG, Balazs Z, Schweizer RA, Nashev LG, Schuster D, Langer T. **Why is 11beta-hydroxysteroid dehydrogenase type 1 facing the endoplasmic reticulum lumen? Physiological relevance of the membrane topology of 11beta-HSD1.** Mol Cell Endocrinol. 2006 Mar 27;248(1-2):15-23.
- Ohyama K, Suzuki M, Kikuchi J, Saito K, Muranaka T. **Dual biosynthetic pathways to phytosterol via cycloartenol and lanosterol in Arabidopsis.** Proc Natl Acad Sci U S A. 2009 Jan 20;106(3):725-30.
- Paterson JM, Seckl JR, Mullins JJ. **Genetic manipulation of 11beta-hydroxysteroid dehydrogenases in mice.** Am J Physiol Regul Integr Comp Physiol. 2005 Sep;289(3):R642-52.
- Picard D, Schena M, Yamamoto KR. **An inducible expression vector for both fission and budding yeast.** Gene. 1990;86(2):257-61.
- Rask E, Walker BR, Söderberg S, Livingstone DE, Eliasson M, Johnson O, Andrew R, Olsson T. **Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11beta-hydroxysteroid dehydrogenase type 1 activity.** J Clin Endocrinol Metab. 2002 Jul;87(7):3330-6.
- Rattanathongkom A, Lee JB, Hayashi K, Sripanidkulchai BO, Kanchanapoom T, Hayashi T. **Evaluation of chikusetsusaponin IVa isolated from Alternanthera philoxeroides for its potency against viral replication.** Planta Med. 2009 Jun;75(8):829-35.
- Rhen T, Cidlowski JA. **Antiinflammatory action of glucocorticoids--new mechanisms for old drugs.** N Engl J Med. 2005 Oct 20;353(16):1711-23
- Ritler D. **Directed evolution of beta-amyrin synthase genes and lupeol synthase genes for expression of chimeric triterpenoid synthase genes in yeast.** University of Applied Sciences and Arts Northwestern Switzerland. School of Life Sciences, 2013 Jul.
- Robinzon B, Michael KK, Ripp SL, Winters SJ, Prough RA. **Glucocorticoids inhibit interconversion of 7-hydroxy and 7-oxo metabolites of dehydroepiandrosterone: a role for 11beta-hydroxysteroid dehydrogenases?** Arch Biochem Biophys. 2003 Apr 15;412(2):251-8.
- Rogers B, Decottignies A, Kolaczowski M, Carvajal E, Balzi E, Goffeau A. **The pleiotropic drug ABC transporters from Saccharomyces cerevisiae.** J Mol Microbiol Biotechnol. 2001 Apr;3(2):207-14.

- Ross J, Li Y, Lim E, Bowles DJ. **Higher plant glycosyltransferases**. *Genome Biol.* 2001;2(2).
- Saleem M, Nazir M, Ali MS, Hussain H, Lee YS, Riaz N, Jabbar A. **Antimicrobial natural products: an update on future antibiotic drug candidates**. *Nat Prod Rep.* 2010 Feb;27(2):238-54.
- Sandeep TC, Andrew R, Homer NZ, Andrews RC, Smith K, Walker BR. **Increased in vivo regeneration of cortisol in adipose tissue in human obesity and effects of the 11beta-hydroxysteroid dehydrogenase type 1 inhibitor carbenoxolone**. *Diabetes.* 2005 Mar;54(3):872-9.
- Sandeep TC, Yau JL, MacLulich AM, Noble J, Deary IJ, Walker BR, Seckl JR. **11Beta-hydroxysteroid dehydrogenase inhibition improves cognitive function in healthy elderly men and type 2 diabetics**. *Proc Natl Acad Sci U S A.* 2004 Apr 27;101(17):6734-9.
- Schuster D, Maurer EM, Laggner C, Nashev LG, Wilckens T, Langer T, Odermatt A. **The discovery of new 11beta-hydroxysteroid dehydrogenase type 1 inhibitors by common feature pharmacophore modeling and virtual screening**. *J Med Chem.* 2006 Jun 15;49(12):3454-66.
- Schweizer RA, Atanasov AG, Frey BM, Odermatt A. **A rapid screening assay for inhibitors of 11beta-hydroxysteroid dehydrogenases (11beta-HSD): flavanone selectively inhibits 11beta-HSD1 reductase activity**. *Mol Cell Endocrinol.* 2003 Dec 30;212(1-2):41-9.
- Seckl JR, Meaney MJ. **Glucocorticoid programming**. *Ann N Y Acad Sci.* 2004 Dec;1032:63-84.
- Segura MJ, Jackson BE, Matsuda SP. **Mutagenesis approaches to deduce structure-function relationships in terpene synthases**. *Nat Prod Rep.* 2003 Jun;20(3):304-17.
- Segura MJ, Meyer MM, Matsuda SP. **Arabidopsis thaliana LUP1 converts oxidosqualene to multiple triterpene alcohols and a triterpene diol**. *Org Lett.* 2000 Jul 27;2(15):2257-9.
- Seki H, Ohyama K, Sawai S, Mizutani M, Ohnishi T, Sudo H, Akashi T, Aoki T, Saito K, Muranaka T. **Licorice beta-amyrin 11-oxidase, a cytochrome P450 with a key role in the biosynthesis of the triterpene sweetener glycyrrhizin**. *Proc Natl Acad Sci U S A.* 2008 Sep 16;105(37):14204-9.
- Seki H, Sawai S, Ohyama K, Mizutani M, Ohnishi T, Sudo H, Fukushima EO, Akashi T, Aoki T, Saito K, Muranaka T. **Triterpene functional genomics in licorice for identification of CYP72A154 involved in the biosynthesis of glycyrrhizin**. *Plant Cell.* 2011 Nov;23(11):4112-23.
- Seki H, Tamura K, Muranaka T. **P450s and UGTs: Key Players in the Structural Diversity of Triterpenoid Saponins**. *Plant Cell Physiol.* 2015 May 6. pii: pcv062.
- Shibuya M, Zhang H, Endo A, Shishikura K, Kushiro T, Ebizuka Y. **Two branches of the lupeol synthase gene in the molecular evolution of plant oxidosqualene cyclases**. *Eur J Biochem.* 1999 Nov;266(1):302-7.
- Stahn C, Löwenberg M, Hommes DW, Buttgerit F. **Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists**. *Mol Cell Endocrinol.* 2007 Sep 15;275(1-2):71-8.
- Stemmer WP. **DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution**. *Proc Natl Acad Sci U S A.* 1994 Oct 25;91(22):10747-51.
- Stewart PM, Murry BA, Mason JI. **Human kidney 11 beta-hydroxysteroid dehydrogenase is a high affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type I isoform**. *J Clin Endocrinol Metab.* 1994 Aug;79(2):480-4.

- Su X, Vicker N, Lawrence H, Smith A, Purohit A, Reed MJ, Potter BV. **Inhibition of human and rat 11beta-hydroxysteroid dehydrogenase type 1 by 18beta-glycyrrhetic acid derivatives.** J Steroid Biochem Mol Biol. 2007 May;104(3-5):312-20.
- Su X, Vicker N, Potter BV. **Inhibitors of 11beta-hydroxysteroid dehydrogenase type 1.** Prog Med Chem. 2008;46:29-130.
- Sun SX, Li YM, Fang WR, Cheng P, Liu L, Li F. **Effect and mechanism of AR-6 in experimental rheumatoid arthritis.** Clin Exp Med. 2010 Jun;10(2):113-21.
- Suzuki M, Xiang T, Ohyama K, Seki H, Saito K, Muranaka T, Hayashi H, Katsube Y, Kushiro T, Shibuya M, Ebizuka Y. **Lanosterol synthase in dicotyledonous plants.** Plant Cell Physiol. 2006 May;47(5):565-71.
- Tannin GM, Agarwal AK, Monder C, New MI, White PC. **The human gene for 11 beta-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization.** J Biol Chem. 1991 Sep 5;266(25):16653-8.
- Tapondjou LA, Ponou KB, Teponno RB, Mbiantcha M, Djoukeng JD, Nguelefack TB, Watcho P, Cadenas AG, Park HJ. **In vivo anti-inflammatory effect of a new steroidal saponin, mannioside A, and its derivatives isolated from *Dracaena mannii*.** Arch Pharm Res. 2008 May;31(5):653-8.
- Taxis C, Stier G, Spadaccini R and Knop M. **Efficient protein depletion by genetically controlled deprotection of a dormant N-degron.** Molecular System Biology. 2009; 5:267.
- Tortorella C, Aragona F, Nussdorfer GG. **In vivo evidence that human adrenal glands possess 11 beta-hydroxysteroid dehydrogenase activity.** Life Sci. 1999;65(26):2823-7.
- Trapp SC, Croteau RB. **Genomic organization of plant terpene synthases and molecular evolutionary implications.** Genetics. 2001 Jun;158(2):811-32.
- Tsigelny I, Baker ME. **Structures important in mammalian 11 beta- and 17 beta-hydroxysteroid dehydrogenases.** J Steroid Biochem Mol Biol. 1995 Dec;55(5-6):589-600.
- Vogt T, Jones P. **Glycosyltransferases in plant natural product synthesis: characterization of a supergene family.** Trends Plant Sci. 2000 Sep;5(9):380-6.
- Vuorinen A, Engeli R, Meyer A, Bachmann F, Griesser UJ, Schuster D, Odermatt A. **Ligand-based pharmacophore modeling and virtual screening for the discovery of novel 17β-hydroxysteroid dehydrogenase 2 inhibitors.** J Med Chem. 2014 Jul 24;57(14):5995-6007.
- Walker EA, Clark AM, Hewison M, Ride JP, Stewart PM. **Functional expression, characterization, and purification of the catalytic domain of human 11-beta -hydroxysteroid dehydrogenase type 1.** J Biol Chem. 2001 Jun 15;276(24):21343-50. Epub 2001 Apr 6
- Wright AP, Gustafsson JA. **Glucocorticoid-specific gene activation by the intact human glucocorticoid receptor expressed in yeast. Glucocorticoid specificity depends on low level receptor expression.** J Biol Chem. 1992 Jun 5;267(16):11191-5.
- Wu TK, Griffin JH. **Conversion of a plant oxidosqualene-cycloartenol synthase to an oxidosqualene-lanosterol cyclase by random mutagenesis.** Biochemistry. 2002 Jul 2;41(26):8238-44.

- Xu R, Fazio GC, Matsuda SP. **On the origins of triterpenoid skeletal diversity.** *Phytochemistry*. 2004 Feb;65(3):261-91.
- Xu R, Sang BC, Navre M, Kassel DB. **Cell-based assay for screening 11beta-hydroxysteroid dehydrogenase inhibitors using liquid chromatography/tandem mass spectrometry detection.** *Rapid Commun Mass Spectrom*. 2006;20(11):1643-7.
- Xue Z, Duan L, Liu D, Guo J, Ge S, Dicks J, ÓMáille P, Osbourn A, Qi X. **Divergent evolution of oxidosqualene cyclases in plants.** *New Phytol*. 2012 Mar;193(4):1022-38.
- Zhang J, Osslund TD, Plant MH, Clogston CL, Nybo RE, Xiong F, Delaney JM, Jordan SR. **Crystal structure of murine 11 beta-hydroxysteroid dehydrogenase 1: an important therapeutic target for diabetes.** *Biochemistry*. 2005 May 10;44(18):6948-57.
- Zhang JH, Dawes G, Stemmer WP. **Directed evolution of a fucosidase from a galactosidase by DNA shuffling and screening.** *Proc Natl Acad Sci U S A*. 1997 Apr 29;94(9):4504-9.
- Zhao H, Arnold FH. **Optimization of DNA shuffling for high fidelity recombination.** *Nucleic Acids Res*. 1997 Mar 15;25(6):1307-8.

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Resume

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Professional experiences

- 11.2011- 11.2015 **PhD thesis**, University of Basel (Biozentrum) and School of Life Science, Institute for Chemistry and Bioanalytics, FHNW Basel.
Subject: Yeast-based strategy for identification of 11 β -HSD1 inhibitors.
- 11.2011- 11.2015 **Scientific collaboration** with the biotech company Evolva SA (Duggingerstrasse 23, 4153 Reinach).
- 10.2009- 07.2010 **Master Thesis**, School of Life Science, Institute for Chemistry and Bioanalytics, FHNW Basel.
Subject: Development of an event-specific assay for the qualitative and quantitative detection of the genetically modified flax CDC Triffid (FP967).
- 03.2008-06.2008 **Bachelor Thesis**, Department of Cellular and Development Biology, University of Studies of Palermo, Italy. Subject: Cloning of the 3'UTR COX4 in the transcription vector pBluescript SK+.

Publications and Patents

- 03.2016 Rosario Vanella, Roberta Callari, Anna Weston, Harald Heider, Markus S. Schwab, Eric Kübler. **Yeast-based assays for screening 11 β -HSD1 inhibitors**. *Microbial Cell Factories* **2016**, 15:52
- 11.2015 Rosario Vanella, Roberta Callari, Eric Kübler, Harald Heider, Markus Schwab. **Yeast-based assays for screening 11 β -HSD1 inhibitors**. (Provisional Patent n. 62251474)
- 01.2014 Rosario Vanella, Anna Weston, Peter Brodmann, Eric Kübler. **Development of an event-specific assay for the qualitative and quantitative detection of the genetically modified flax CDC Triffid (FP967)**. *Food Control* **2014**, 41, 128-133

Education

- 11.2015 **PhD in Biochemistry**,
University of Basel, “Magna cum Laude”
- 03.2011 **Master of Science Diploma in Biotechnology**
University of Studies of Palermo, “Summa cum Laude”
- 07.2008 **Bachelor of Science Diploma in Biotechnology**
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International Experiences

- 11.2011- 11.2015 **PhD thesis**, University of Basel (Biozentrum) and School of Life
Science, Institute for Chemistry and Bioanalytics, FHNW Basel.
- 10.2009-07.2010 **Erasmus Programme:** Master Thesis position at FHNW, Basel.

Conference Presentations

- 09.2014 **9th International Summer School on Advanced Biotechnologies**
(Palermo, Italy) **Speaker:** Synthetic biology for the identification of
novel 11 β -HSD1 inhibitors in yeast.
- 09.2011 **6th International Summer School on Advanced Biotechnologies**
(Santa Margherita del Belice, Italy) **Speaker:** Development of an
event-specific assay for the qualitative and quantitative detection of
the genetically modified flax CDC Triffid (FP967).

Working Skills

Molecular Biology: PCR, qPCR, recombinant DNA technology,
mutagenesis, sequencing

Biochemistry: SDS electrophoresis, western blot, electrophoretic
mobility shift assay, biochemical assays

Bacterial and yeast biology: plasmid transformation, growth and
survival curves, heterologous genes expression, manipulation and
tetrad dissection, mating type switch

Scientific instruments: FACS, fluorescence microscopy, HPLC and
GC-MS

Computer Skills: Experience with several software in bioinformatics
and with MS Office tools.

Languages

Italian (mother tongue), **English** (fluent), **German** (basic)

Referees

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Rosario Vanella

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