screen that selected domains causing almost complete removal of the fusion protein. TMP stabilizes the DD-target protein fusion in a dose-dependent manner up to 100-fold, which gives the system a substantial dynamic range. Stabilization is reversible and removal of TMP leads to rapid degradation back to background levels. In addition, the ligand TMP works by itself and does not require dimerization with a second protein, similar to an earlier version developed by the same group (Banaszynski et al., 2006). This system appears to be so effective that it can even control the levels of transmembrane proteins, which greatly increases its potential utility and applicability.

The stabilizing ligand used for this system also brings some important practical advantages. TMP is commercially available, inexpensive, and has good pharmacological properties, which makes its usage in in vivo experimental systems easier. TMP has very few off-target effects in mammalian cells because it inhibits ecDHFR much more strongly than the endogenous mammalian DHFR. In fact, it is used as an antibiotic. Additionally, TMP crosses the blood-brain barrier and can therefore be used to modulate the stability of proteins in the central nervous system of live animals. Finally, TMP also crosses the placental barrier, which should permit the study of proteins that are essential in the earliest stages of development. If the offspring contain a gene modified with ecDHFR, TMP could be administered to the mother throughout gestation to ensure the presence of the protein of interest. At the desired times, TMP could then be withdrawn so that the target protein is degraded. This would make it possible to investigate the role of the targeted protein at various stages of development.

Thus, the new ecDHFR-derived DD method represents a substantial step forward for inducible protein degradation systems, and it could become a practical addition to our tools for regulating protein concentrations.

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Creating Designer Laccases

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High redox potential laccases from white-rot fungi are recalcitrant to engineering. Maté et al. (2010) employed directed evolution to improve the activity and expression level of the fungal laccase from basidiomycete PM1, followed by rational design to restore thermostability lost during evolution, resulting in a highly active and stable enzyme.

Highly active and stable enzymes are desirable for industrial biotechnology. Two methods are commonly used to obtain enzymes with such properties: screening microorganisms to identify novel enzymes, and engineering existing enzymes to improve these properties. While traditional strain screening is still widely used, enzyme engineering, in particular directed-enzyme evolution, has increasingly become an alternative to generate enzymes with desired properties. This is particularly true when enzyme properties, such as enzymatic activity and thermal stability, can be analyzed in a high throughput manner to facilitate the identification of superior mutants from fairly large mutant libraries. In a typical directed-evolution experiment, screening for enhanced enzymatic activity is performed concomitantly with screening for improved thermostability to generate mutants that are both more active and more stable. With such a procedure, some mutants with enhanced activity but less stability are obtained, while others with enhanced stability but less activity are also frequently identified. If the additive mutations fail to be combined, either the more active mutant or

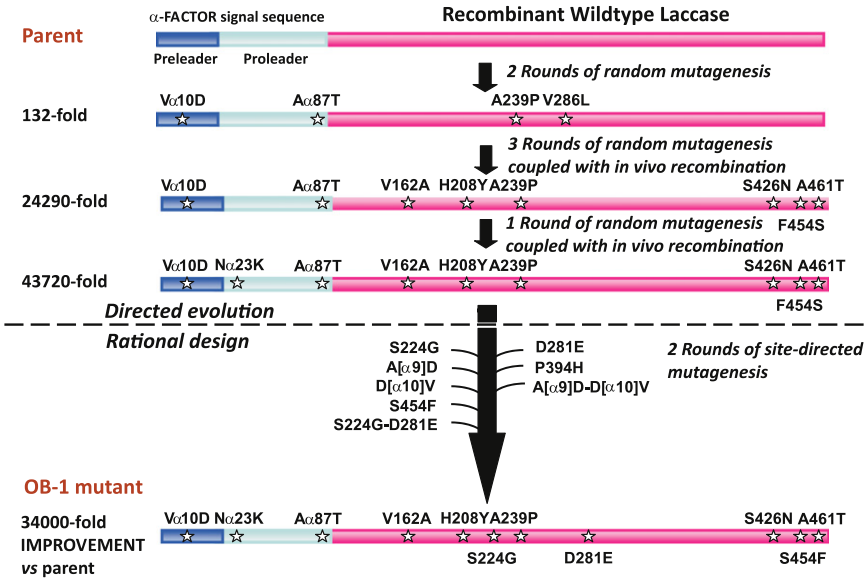

Figure 1. Joint-Directed Evolution of the α-Factor Pre-Proleader and a High Redox Potential Laccase

A combination of different evolutionary approaches (random mutagenesis, in vivo DNA shuffling, and IvAM) was employed in the α-PM1 fusion gene. From the sixth generation, rational design strategies (site-directed mutagenesis for both beneficial mutation recovery and mutational exchange with an evolved HRPL homolog) were carried out to recover the decrease in thermostability that resulted from the accumulation of mutations during evolution and to further enhance activity. Synonymous mutations are not shown. This figure was provided by Dr. Miguel Alcalde.

the more stable one is used for the next round of directed-evolution experiments, causing the loss of beneficial mutations in the protein fitness landscape. In this issue, Maté et al. (2010) employed site-directed mutagenesis to introduce mutations unable to be recombined, and rational design to restore reduced thermostability of a highly active mutant laccase generated by directed evolution (Figure 1), resulting in a highly active and stable enzyme with promising applications in industry.

Laccases are multi-copper oxidases using oxygen to oxidize various phenols and nonphenol compounds, with water as the exclusive side product (Singh Arora and Kumar Sharma, 2010). Laccases possessing high redox potentials at their T1 copper sites are classified as high redox potential laccases (HRPLs), and are of great interest for industrial applications in pulp bleaching, food processing, textile treatments, and bioelectrochemistry, due to their relatively relaxed substrate specificity (oxidize higher redox potential compounds) and thermostability (optimal temperature as high as 80°C) (Singh Arora and Kumar Sharma, 2010; Hilden et al., 2009). To engineer a laccase with directed evolution, it is first necessary to functionally express the laccase in microorganisms that are amenable to genetic manipulations, such as the yeast *S. cerevisiae* or the bacterium *E. coli*. Because thermally stable fungal laccases are glycosylated with carbohydrate contents as high as 50%, *S. cerevisiae* has been the preferred microorganism for functional expression in order to maintain glycosylation. Recombinant expression in yeast has allowed for the directed evolution of standard laccases for improved total activity (both specific activity and expression level), altered substrate specificity, and enhanced thermostability (Rodgers et al., 2009). However, such reports are limited for HRPLs, due to their poor functional expression in yeast. To overcome this limitation, Maté et al. (2010) started with the highly stable HRPL basidiomycete PM1 (optimal temperature is 80°C) and fused it to the native leader sequence of the yeast α-factor mating pheromone. This fusion resulted in PM1 being secreted from the cell with poor but sufficient activity for developing a high-throughput colorimetric screen for the directed evolution of total laccase activity (specific activity, secretion, and expression levels). In the first two rounds of directed evolution, random mutagenesis libraries were created by the most common method, error-prone PCR, using the wild-type recombinant α-PM1 or the most active mutant from the first generation as templates. In general, in vitro recombination methods, including DNA shuffling and StEP, are used to recombine beneficial mutations when multiple mutants are identified in initial generations (Stemmer, 1994; Zhao et al., 1998). Instead, Maté et al. (2010) took advantage of the highly efficient in vivo recombination machinery of yeast to generate recombined mutants from the random mutagenesis libraries for the third and subsequent rounds of directed evolution. After five generations, mutant 7H2 was obtained with more than a 24,000-fold improvement in total activity, though the thermostability of the mutant was also significantly decreased. By screening thermostability and total activity simultaneously in the sixth generation starting from 7H2, two mutants were obtained that had either increased activity (6C8) or thermostability (16B10), but not both. Rather than continuing to evolve the enzyme toward either total activity or thermostability starting from the corresponding mutant, two clever strategies were applied from this point to recover the lost thermostability and further enhance total activity. First, by analyzing the structural information of a homologous laccase, Maté et al. (2010) hypothesized that a mutation introduced in the fifth generation (7H2) was responsible for the reduced thermostability. Reverting the mutated amino acid residue F454S in mutant 6C8 (the best activity variant from sixth generation) to the wild-type using site-directed mutagenesis resulted in a mutant with similar activity to 7H2 and completely restored thermostability. Second, by reintroducing beneficial mutations identified in the first round of mutagenesis, and those which were lost during recombination in the later rounds due to their close proximities on the DNA sequence, mutant OB-1 was generated. Without a priori knowledge of their impacts on thermostability, these beneficial mutations contributed significantly to the total enzyme activity (approximately a 34,000-fold improvement from the wild-type). An additional mutation gleaned from another engineered HRPL further

enhanced activity but destabilized the enzyme, and was not incorporated into the final mutant OB-1. There are 15 mutations in OB-1, including five (two synonymous) in the α-factor leader sequence, that collectively contribute to both the expression/secretion level and specific activity while retaining wild-type thermostability. As such, mutant OB-1 is more thermally stable than some native thermostable fungal laccases and also highly resistant to organic solvents and acidity, making it an excellent candidate for industrial applications. In addition, the general nature of the mutant leader peptide is likely to enhance secretion levels when used with other proteins.

Laboratory evolution of highly active enzymes tends to cause the engineered enzymes to lose activity toward substrates not used during screening, a consequence that may result from the altered shapes of enzyme active sites. To maintain the broad substrate specificity of PM1 laccase, Maté et al. (2010) employed two representative substrates in their screen. While such a strategy is not always effective in maintaining broad substrate specificity, the resulting mutant OB-1 exhibits increased specific activity toward all three substrates examined with only a slight preference for ABTS rather than guaiacol when compared with the third-generation mutant. In some applications, such as delignification, a chemical mediator is usually used to indirectly oxidize phenol and nonphenol compounds (Call and Mucke, 1997). As such, a highly active laccase toward a chemical mediator (for example, 1-hydroxybenzotriazole for delignification) would eliminate the necessity of maintaining broad substrate specificity in laccase-directed evolution. In addition, the inadvertent introduction of destabilizing mutation F454S could also have been circumvented if a thermal stability screen were incorporated into the initial library screening, though such a screen may not have been feasible in the presence of poor total enzymatic activity. Nevertheless, this fine piece of work represents another interesting example of how directed evolution and rational enzyme design can be combined for the engineering of protein function, and how such a strategy could enhance the efficiency of searching protein sequence space (Arnold, 2006; Turner, 2009). More immediately, Maté et al. (2010) have developed a heterologous expression system for efficiently producing HRPLs, which has been a major hurdle for industrial applications of laccases, and the highly active and stable OB-1 is an excellent starting point for various biotechnological applications of HRPLs.

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A Group I Intron Riboswitch

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Riboswitches are mRNA-based elements that regulate gene expression via binding of a specific ligand. Breaker and coworkers have now discovered a novel type of regulatory RNA motif that acts by c-di-GMP-dependent control of a self-splicing group I intron ribozyme (Lee et al., 2010).

Riboswitches are widespread regulatory motifs found in mRNAs. They are usually composed of an aptamer domain facilitating ligand binding and a second domain termed an expression platform (Roth and Breaker, 2009). The latter reorganizes in response to ligand binding to the aptamer domain, resulting in altered gene expression. Commonly one out of two different mechanisms is employed: either the formation of a Rho-independent termination structure is regulated, resulting in the premature termination of transcription, or the formation of a stem-loop structure that masks the ribosome binding site is controlled, resulting in the regulation of translation initiation. Exceptions are represented by riboswitches operating in eukarya, as well as an autocatalytic motif (the *glmS* riboswitch in certain eubacteria) that cleaves the mRNA in response to glucosamine-6-phosphate binding (Winkler et al., 2004).

Now Breaker and coworkers have described a novel type of riboswitch that contains a self-splicing ribozyme as an expression platform (Lee et al., 2010).

