# SYNOPSIS OF THE THESIS

## Title: Rational elicitation of cold-sensitive phenotypes

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Advances in genome sequencing and cloning techniques have made the information about genes and their products accessible. Yet, this information is physiologically relevant only when it can be supplemented by *in vivo* studies. Every organism expresses genes selectively at any given time. Regulated gene expression in viruses helps make the decision about switching from lysogeny to lysis. In bacteria, it aids in making choices about carbon sources, helps to best utilize existing amino acids rather than synthesize them, regulates cell division and might even play a role in persistence. Controlled and orchestrated gene expression in higher organisms drives developmental processes, cell signaling, cell division, immune responses etc. The understanding of organismal biology, thus, not only involves the study of the sequence in which different genes are expressed but also the manner in which their expression is regulated.

It is desirable to have a means to modulate the expression of specific genes and to control the activity of their protein products *in vivo*, so as to be able to study their effects on the cell. This can be achieved by means of conditional gene

expression and conditional mutants. Conditional mutants are functional under one set of conditions, termed 'permissive', while under other conditions which are 'restrictive', they become non-functional. The wild-type (Wt) is functional under both these conditions. Conditional mutants are especially useful for studying the essential or lethal genes in an organism. Temperature-sensitive (heat-sensitive) and cold-sensitive mutants are one such class of conditional mutants, wherein the restrictive and permissive conditions are obtained by varying the temperature. By using temperature shift as a condition, the target gene function can be modulated easily, rapidly, reversibly and selectively. Temperature Sensitive (ts) mutants of a gene are ones in which there is a marked drop in the level or activity of the gene product when the gene is expressed above a certain temperature (non-permissive or restrictive temperature). Below this temperature (permissive temperatures), the phenotype of the mutant is very similar to that of the wild type. On the other hand, cold sensitive (cs) mutants behave like loss-of-function mutants at low temperatures, but have wild type like phenotypes at higher temperatures. Cold-sensitive phenotypes have been used to understand macromolecular assembly and biological phenomena such as ribosome assembly and the cell cycle, yet few attempts have been made to understand the basis of cold sensitivity or to elicit it by design. **Chapter 1** of this thesis, the introductory chapter, gives a brief overview of conditional gene expression and conditional mutants, with emphasis on ts and cs mutants. This chapter also covers relevant literature addressing the probable molecular basis for heat and cold sensitivity, and the attempts at designing such mutants.

Most partial loss-of-function mutants are intrinsically temperature sensitive, since processes such as aggregation and degradation are enhanced at higher

temperatures. There are also several studies that highlight the role played by *in vivo* protein levels in causing ts and cs phenotypes. In the light of these observations, we hypothesized that increasing the expression levels of ts mutants and other partial loss-of-function mutants selectively at higher temperatures could overcome the debilitating effect of the mutation at those temperatures, leading to Wt-like phenotypes at high temperatures, while the meagre basal level expression at lower temperatures could result in a cs phenotype. **Chapter 2** discusses the successful elicitation of cs phenotypes by expressing known partial loss-of-function mutants of *E. coli* CcdB and *S. cerevisiae* Gal4 using heat inducible expression systems. The transferability of the cs phenotype of the Gal4 mutants from yeast to Drosophila is also demonstrated.

Classically, ts and cs mutants have been isolated by laborious screening of random mutant libraries generated by error-prone PCR or by exposure to mutagens. In higher organisms, this process of isolating conditional mutants in a gene of interest can be extremely tedious. Thus, it is desirable to have a means of rationally designing ts and cs mutants. Previous work in the lab has resulted in a methodology for rational design of ts mutants by using the amino acid sequence as the sole input. An algorithm was used to predict buried residues, and it was shown that by mutating these residues, it was possible to obtain ts mutants. **Chapter 3** of this thesis describes how this approach was extended for rational elicitation of cs phenotypes. We have designed partial loss-of-function mutants of yeast Ura3 and Trp1 based solely on the amino acid sequence, cloned these under a heat shock promoter and obtained cs phenotypes. Additional characterization of purified Gal4 mutants by measuring protein thermal stability and DNA binding affinity, as well as measurements of transcript levels by qPCR were carried out, to understand the molecular basis of the phenotype.

Analogous to conditional mutants, conditional gene expression is a tool for selectively turning genes 'on' and 'off' as desired, by means of a trigger such as temperature or a chemical inducer. One such conditional gene expression system is based on the *ara* operon. The *ara* operon consists of the genes involved in uptake and metabolism of arabinose. Along with the *lac* operon, it is one of the most thoroughly studied gene regulation systems. It is tightly repressed by glucose, has low basal expression and shows a graded response upon being induced by varying arabinose concentrations. The pBAD series of vectors, containing the $P_{BAD}$ promoter and the araC regulator-activator, are very convenient for cloning and expression purposes, and have been used extensively in the lab for cloning and phenotypic screening of mutant libraries. However, there have been reports of non-uniform gene expression across cells at sub-saturating concentrations of arabinose. This heterogeneity of gene expression is found to arise as a result of arabinose induced auto-catalytic expression of the arabinose transporters. Heterologous over-expression of the transporter AraE is reported to resolve this issue, however this is still controversial. **Chapter 4** covers studies on this issue of heterogeneity of expression from $P_{BAD}$ promoters at the single cell level, using stable and degradable GFPs as reporters, in a variety of conditions such as constitutive versus autocatalytic expression of transporter, presence and absence of arabinose metabolising *araBAD* genes in the host, and varying time periods of induction.

Several single amino acid substitutions which cause folding defects in the protein are seen to give rise to cs or ts phenotypes. The key to understanding the basis of such phenotypes caused by folding defective mutants lies in the folding pathway of the protein. Previous work in the lab has identified several mutants of *E. coli* CcdB

which have lower *in vivo* activity and solubility when compared to the Wt, but show similar thermal stability *in vitro*. These mutants are proposed to be folding defective. In order to understand the basis of the defect brought about by the mutation, it is necessary to first study the folding mechanism of the Wt CcdB protein. **Chapter 5** deals with studies on the folding kinetics of *E. coli* CcdB. This study looks at the folding of a dimeric protein, which is of great interest as it involves conformational changes as well as association steps. The results from various experimental observations, ligand binding studies and simulations lead to the conclusion that CcdB folds via parallel pathways, each involving an unstructured dimeric intermediate, to arrive at its native state. The **appendix** to this chapter details the studies done to determine the nature and oligomeric status of CcdB at pH 4.