Pre-mRNA splicing is a process, in which non-coding segments, introns, are removed from precursor messenger RNA (pre-mRNA). This process, a critical event in gene expression of nearly all human genes, is highly regulated, and connected with transcription and other processing events. Pre-mRNA splicing is catalyzed by the spliceosome, a large and dynamic macromolecular machine, called the spliceosome. Recent studies by three groups led by R. Luhrmann, K. Nagai, and Y. Shi revolutionized studies in the splicing field by providing a wealth of exciting cryo-EM structural information on various forms of spliceosomes captured at different stages of assembly and catalysis. Based on this information we will analyze several predictions posed by these structures. The long-term, broad goals of this project are to understand interactions and rearrangements between spliceosome components and the RNA substrates leading to catalysis. Investigation of these events will elucidate interactions and rearrangements among core components of the spliceosome, allowing for a better description of its function and ultimately helping to predict the outcome of splicing patterns.

This proposal focuses on mechanisms by which three elements within the spliceosome: the catalytic triplex, a region of Prp8 protein in proximity of the active site and the N-terminal domain of Cwc25 protein, impact the function of the catalytic center. Although cryo-EM structures of the spliceosome view its catalytic core as unchanging, yeast genetic analysis of the spliceosome and structural studies of group II introns suggest that the catalytic center may undergo significant conformational changes during the catalytic phase. Our experiments will investigate if the catalytic triplex, an essential component of the catalytic center, undergoes conformational rearrangements during splicing. Following an example of group II introns, we will test multiple combinations of nucleotide interactions implicated in the formation of the catalytic triplex and test their functionality in vivo. This will help to elucidate the scope of dynamic changes at the catalytic center, possibly leading to a careful re-evaluation of the current model of the second step catalytic core. We will also analyze the consequences of changes at the catalytic triplex that favor the first or the second step on the global splicing profile of yeast transcriptome (Aim 1).

Secondly, we will analyze the contributions of a conserved region of Prp8 protein in proximity of the active site, which based on the cryo-EM structures may bring together several components of the catalytic center. This segment encompasses several newly identified alleles that seem to affect splicing catalysis rather than the transition between the two catalytic reactions, representing a mechanistically new class of mutants affecting splicing. We will analyze the involvement of this Prp8 region in splicing, testing the possibility that it coordinates different components of the catalytic center and juxtaposes them for catalysis (Aim 2).

Finally, we will address one of the basic, yet still not clearly understood questions concerning the selection and positioning of one of the reactants in the first splicing reaction: the branch site Adenosine (BS-A). The BS motif is recognized by pairing to the complementary region in U snRNA, and the BS-A bulges out of this duplex. However, it is not clear how is it determined which nucleotide bulges out, specifically, which of the two consecutive A residues is selected as the BS-A. To better understand the selection and positioning of the BS-A for catalysis, we will focus on the N-terminal domain of Cwc25 protein, which contacts the U2:BS duplex. In particular, we will ask if a negatively charged *cwc25-D5* residue is involved in specifying the BS-adenosine. In parallel, we will investigate the previously suggested role of pseudouridine modification at U2 pos. 35 in this process. These studies will be facilitated by the previously established orthogonal system, in which the U2:BS sequences can be freely changed, allowing for detailed analysis of the mechanism of branch site selection (Aim 3).

Our studies are based on the recent information provided by cryo-EM structures and will validate their predictions, expanding structural information with a more detailed, functional analysis of several fundamental aspects of splicing reactions. In humans, expression of nearly all mRNA transcripts involves pre-mRNA splicing. Typically, human pre-mRNA transcripts undergo a complicated type of splicing, so called alternative splicing, in which many different forms of mRNA can be generated form a single pre-mRNA molecule. I am convinced that if we want to better understand this process and be able to predict the complex alternative splicing patterns, we need the detailed information from mechanistic, functional studies, like those proposed in this project.