Junior Fellow/Principal Investigator:

Type 1 diabetes (T1D) is characterized by the selective destruction of insulin-secreting pancreatic β-cells by autoreactive immune cells.[1,2] These cells produce pro-inflammatory cytokines induce β-cell death. Described below are my two key findings pertaining to T1D.

1) Lineage reprogramming of α-cells to insulin-producing cells by targeting druggable proteins:[3] Lineage reprogramming of other pancreatic cell types to beta cells can compensate for insulin deficiency in T1D models and has been accomplished by overexpression of master-regulatory transcription factors.[4,5] The therapeutic promise of these discoveries is limited by our inability to target "undruggable" transcription factors and to deliver them in cells. Two small-molecule kinase inhibitors, BRD7389 and GW8510, were known to induce beta cell-like characteristics (e.g., insulin expression) in pancreatic alpha cells.[6,7] Hence, I hypothesized that the downregulation of specific phosphorylated proteins can induce insulin expression in alpha cells. Specifically, I was interested in proteins whose phosphorylation levels were lower in beta cells and in small molecule-treated alpha cells compared to that in alpha cells. To identify such proteins, we performed a global quantitative phosphoproteomics experiment using stable isotope labeling by amino acids in cell culture (SILAC) for three conditions (Fig. 1). From these proteins, we identified two kinases, Brsk1 and Cammkk2, whose knockdown induced lineage reprogramming of alpha cells to produce insulin and other key markers of beta cells. Small molecules that target these kinases were also able to induce insulin expression in alpha cells. Thus, my efforts led to new "druggable" targets that might enable reprogramming of alpha cells to insulin-producing cells in T1D patients.

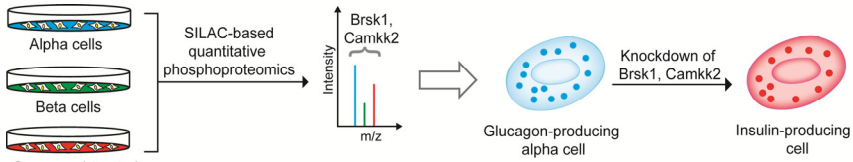

Fig. 1. Reprogramming of α-cells to insulin-producing cells by targeting kinases.

2) Targeting the ubiquitin system to block cytokine-induced dysregulated JAK-STAT signaling:[8] Dysregulated JAK-STAT signaling is a key component of cytokine-induced beta cell apoptosis in T1D. My interest to identify targets for blocking cytokine-induced beta cell apoptosis led me to the small molecule BRD0476. This small molecule inhibited cytokine-induced beta cell apoptosis, but its underlying mode-of-action was unknown.[9] Using pulldown and biophysical studies, we discovered that BRD0476 bound to the deubiquitinase, USP9X, which increases half-lives of key signaling proteins by removing their ubiquitin chains.[10,11] Furthermore, gene-expression analysis showed that BRD0476 blocks the JAK-STAT pathway. Interestingly, BRD0476 did not inhibit the kinase activity of JAK, unlike the extant JAK-STAT inhibitors, indicating that BRD0476 was targeting a heretofore unknown regulatory element of that pathway. Motivated by these interesting findings, I continued my efforts to delineate the relationship between USP9X and the JAK-STAT pathway. The initiation of JAK-STAT pathway involves JAK phosphorylation at Y1007/1008. This phosphorylation also signals rapid ubiquitination of JAK by recruiting ubiquitin-ligases (Fig. 2).[12] I hypothesized that sustainment of JAK-STAT signaling must require rapid JAK deubiquitination by a deubiquitinase. Furthermore, I hypothesized that inhibition of such a deubiquitinase would block JAK-STAT pathway by increasing levels of signaling incompetent JAK. Using BRD0476 and reverse chemical genetics, I have now confirmed these hypotheses and confidently identified the deubiquitinase as USP9X. Thus, I have delineated a previously unknown regulatory control of the JAK-STAT pathway by the ubiquitin system. Furthermore, my efforts have opened a new therapeutic avenue to target dysregulated JAK-STAT signaling in many diseases.

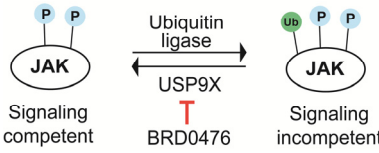

Fig. 2. Targeting USP9X to block dysregulated JAK-STAT signaling.

Doctoral Research:

In 1951, Linus Pauling laid the foundation of structural biology by describing the existence of hydrogen bonds in proteins.[13] My Ph. D. thesis describes the discovery of another force, termed the $n \rightarrow \pi^*$ interaction, that is akin to the hydrogen bond. The quantum-mechanical kinship between hydrogen bonds and $n \rightarrow \pi^*$ interactions is evident in the very structure used by Pauling to describe hydrogen bonds—an α-helix (Fig. 3).[14] The canonical hydrogen bond involves delocalization of the lone pair (n_s) of the

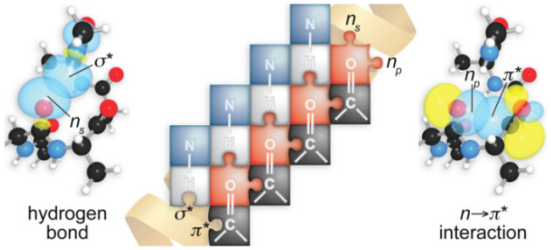

Fig. 3. Kinship of H-bonds and $n \rightarrow \pi^*$ interactions in an α-helix.

carbonyl's oxygen into the N–H antibonding orbital (σ*). An $n\rightarrow\pi^*$ interaction, however, involves the delocalization of the second carbonyl lone pair (n_p) into the antibonding orbital (π^*) of the adjacent carbonyl.

My initial studies established the two signatures of an $n\rightarrow\pi^*$ interaction. First, the $n\rightarrow\pi^*$ interaction induces a short contact between the interacting groups, wherein their van der Waals surfaces interpenetrate (Fig. 4A, 4B). Second, it deforms an otherwise planar carbonyl group to a pyramidal geometry (Figure 2C). This signature is reminiscent of the Bürgi–Dunitz trajectory for nucleophilic additions to carbonyl groups. These signatures can be detected by X-ray crystallography and quantum-mechanical calculations.[15-18] Next, I established the pyramidalization signature of $n\rightarrow\pi^*$ interactions in α-helices.[19] I used atomic resolution crystal structures of helical peptides containing both α- and β-amino acid residues. The α-amino acid residues, which adopt the α-helical conformation, displayed short contact and dramatic pyramidalization, but the β-amino acid residues did not display these signatures. Thus, β-amino acid residues provided an internal control to unequivocally establish the signatures of $n\rightarrow\pi^*$ interactions in α-helices.

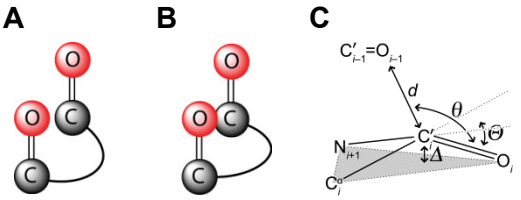

Fig. 4 (A) Carbonyl groups without $n\rightarrow\pi^*$ interaction and short contact (B) Carbonyl groups with $n\rightarrow\pi^*$ interaction and short contact (C) A carbonyl group pyramidalized by $n\rightarrow\pi^*$ interaction.

Motivated by these findings, I computationally searched for the signatures of $n\rightarrow\pi^*$ interactions in the allowed regions of the Ramachandran map. My computational studies not only indicated widespread occurrence of $n\rightarrow\pi^*$ interactions in these regions, but also suggested significant stabilization of common secondary structures such as α-, 3_{10}-, and polyproline II helices, and twisted β sheets by these interactions (Fig. 5A).[14] These observations were validated by statistical analyses of high-resolution protein structures in the Protein Data Bank (Fig. 5B). ***The short contact signature of n→π* interactions was observed in nearly every protein*** and is present in numerous protein–ligand complexes.

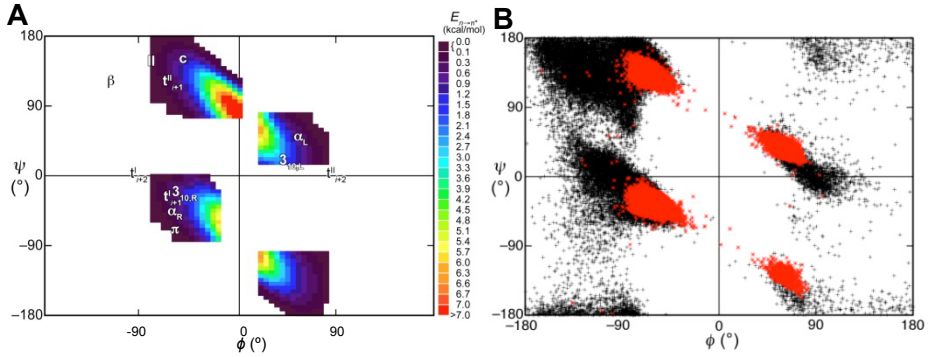

Fig. 5. (A) Common secondary structures with $n\rightarrow\pi^*$ interaction. (B) Regions of Ramachandran Map with (red) and without (black) $n\rightarrow\pi^*$ interaction.

I next embarked on validation of the hypothesis that $n\rightarrow\pi^*$ interactions stabilize the aforesaid secondary structures. I envisioned that deletion of $n\rightarrow\pi^*$ interactions in these structures would be deleterious to their conformational stability. Towards this goal, we utilized a carbonyl group mimic that does not partake in $n\rightarrow\pi^*$ interaction despite having similar geometrical properties. We and others found that deletion of an $n\rightarrow\pi^*$ interaction indeed reduced the conformational stability of several secondary structures, including α-, 3_{10}-, and polyproline II helices, and β-turns.[21-25,18,26] For example, the preference for the all *trans* conformation in β-turns was lost upon deletion of a single $n\rightarrow\pi^*$ interaction (Fig. 6).

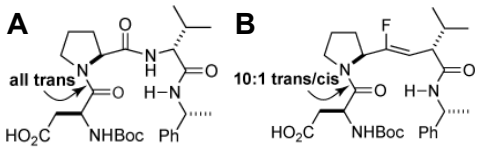

Fig. 6. (A) A canonical β-turn with all trans conformation. (B) A disrupted β-turn on deletion of $n\rightarrow\pi^*$ interaction

Structural and Functional roles of $n\rightarrow\pi^*$ Interaction:

<u>$n\rightarrow\pi^*$ Interaction in Proteins:</u>[14] The $n\rightarrow\pi^*$ interaction, by stabilizing common secondary structures, plays an important role in protein stability. Interestingly, amplification of $n\rightarrow\pi^*$ interaction in proteins can increase their stability. By amplifying this force, Raines and co-workers were able to engineer the most stable collagen ever made.[27] Currently, these engineered hyperstable collagens are in preclinical trials as wound-healing agents. In addition to protein stability, $n\rightarrow\pi^*$ interactions facilitate protein folding by stabilizing the incipient structures in the nucleated or biased unfolded states (Fig. 7). On the other hand, $n\rightarrow\pi^*$ interactions can endow undesirable conformational attributes to a

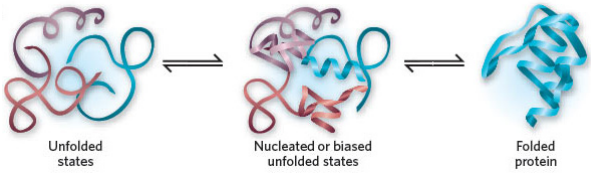

Fig. 7. $n\rightarrow\pi^*$ interactions assists protein folding by populating the nucleated or biased folded states.[20]

molecule. This is presumably the case with the 4*S* diastereomer of hydroxyproline and this might explain its absence from our proteome.[28] In addition to contributing to the thermodynamic stability of proteins, our data indicates that $n\rightarrow\pi^*$ interactions contribute to the chemical stability of proteins by making the amide bonds resistant to hydrolysis.[29] We have also shown that $n\rightarrow\pi^*$ interaction can convert an otherwise planar, achiral peptide bond to a pyramidal and chiral entity.[30] This induction might play an important role in bio-molecular recognition processes.

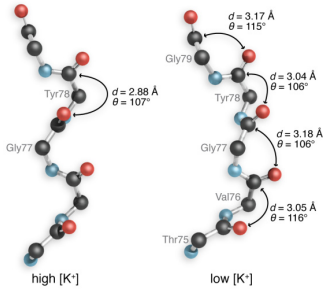

Fig. 8. $n\rightarrow\pi^*$ interactions in K^+ channel.

<u>$n\rightarrow\pi^*$ Interaction in K^+ channel:</u>[14] The selectivity filter in potassium channel adopts different conformations at low and high concentrations of K^+ ion. The filter is open at a high [K^+] and is closed at a low [K^+]. At a high [K^+], the carbonyls coordinate to dehydrated potassium ions, which prevents them from engaging in $n\rightarrow\pi^*$ interaction. However, upon depletion of K^+ a conformational switch occurs to a non-conducting conformation where most of the residues engage in an $n\rightarrow\pi^*$ interaction (Fig. 8). $n\rightarrow\pi^*$ interactions may drive the attainment of the non-conducting conformation and closure of the K^+ channel.

<u>$n\rightarrow\pi^*$ Interaction in fluorescent proteins:</u>[31] Green Fluorescent Protein (GFP) and its analogs have revolutionized biological imaging. The chromophore of these fluorescent proteins fluoresces only when it is present in the very rigid and tightly packed protein environment. In the absence of a rigid environment, non-radiative decay occurs by rotation across the double bond shown in fig. 9A. The high-resolution crystal structures of fluorescent proteins indicate that the residue that engages in an $n\rightarrow\pi^*$ interaction with chromophores embraces the chromophore and provides the rigid, tightly packed environment (Fig. 9B). Additionally, chromophore biogenesis involves intermediates that are stabilized by this interaction.

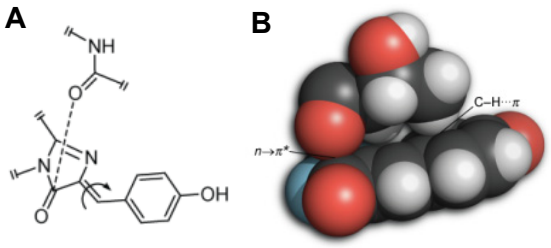

Fig. 9. (A) Non-radiative decay due to rotation across the double bond. (B) $n\rightarrow\pi^*$ interactions provide rigid milieu to GFP's chromophore.

<u>$n\rightarrow\pi^*$ Interaction in Aspirin:</u>[32] The existence of an $n\rightarrow\pi^*$ interaction in the common household drug, Aspirin, has been validated both computationally and experimentally. Aspirin relieves pain by chemically modifying the serine residue located near the active site of cyclooxygenases. To accomplish this remarkable feat, aspirin, which is hydrophilic, must traverse through a hydrophobic channel. By shielding aspirin's negative charge, $n\rightarrow\pi^*$ interaction, imparts hydrophobicity to aspirin and facilitates its passage through the hydrophobic channel. The use of $n\rightarrow\pi^*$ interaction as a general strategy for delivery of very polar drug molecules in cells is currently in progress.

<u>$n\rightarrow\pi^*$ Interaction in the Origin of Life:</u>[33] According to the RNA world hypothesis, the first informational polymer was RNA. The pre-biotic synthesis of RNA building blocks defies the fundamental stability and reactivity rules of organic chemistry. Interestingly, the unusual stability and reactivity of the building block was key to successful RNA synthesis. X-ray crystallographic analysis and kinetic studies suggest that an $n\rightarrow\pi^*$ interaction contributed to this unusual stability and reactivity. Thus, an $n\rightarrow\pi^*$ interaction may have opened the gateway to the "RNA world".

In summary, my Ph. D. research describes the discovery of a hydrogen bond-like interaction in biologically important small-molecules, proteins, and protein–ligand complexes. This discovery has furthered our fundamental understanding of protein folding, stability, and function. Additionally, it has spurred several applications in the areas of nanomaterial and drug design, catalysis, and biomimetic chemistry.

Pre-Doctoral Research:

My pre-doctoral research primarily involved the total synthesis of natural products and biophysical studies to understand protein folding. My M.S. thesis describes the stereoselective synthesis of (–)-microcarpalide (Fig. 10, **1**) and general enantioselective routes to 1,2-aminoalcohols. Starting with different diastereomers of tartaric acid, I synthesized the intermediates **2** and **3** in eleven and nine steps respectively (Fig. 10). The key step in the synthesis of intermediate **2** involved a chelation-controlled addition of vinylmagnesium bromide to an α-benzyloxy aldehyde while an analogous chelation-controlled Keck allylation was used to generate intermediate **3**. The other key step in their synthesis involved a stereoselective reduction of C_2-symmetric

diketones with L-Selectride.[34] In addition to the synthesis of microcarpalide, these α-hydroxy aldehydes were extrapolated to several 1,2-aminoalcohols.

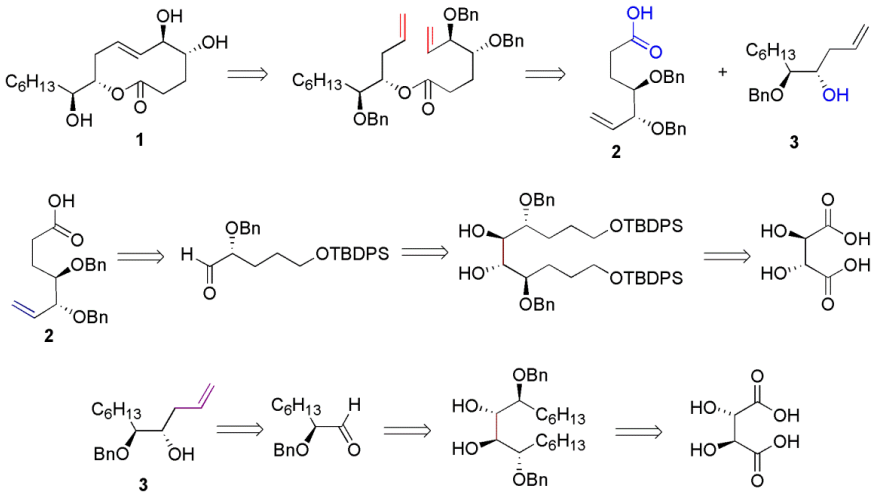

Fig. 10. Chiral pool approach for synthesis of (–)-microcarpalide

I also performed preliminary studies to determine the specific and non-specific components in the early events of protein folding. Our rationale was to monitor the changes in protein's intramolecular distances during the early stages of protein folding using Fluorescence Resonance Energy Transfer (FRET). To this end, I performed site-directed mutagenesis to generate several single cysteine mutants. The cysteine residues were then labeled with a dye, which formed a donor-acceptor FRET pair with tryptophan in the protein. The changes in distance between the dye and the tryptophan residue during the initial, sub-milliseconds of protein folding were measured by FRET.[35]

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