**Objectives**:

*Candidozyma auris* has emerged as a significant global health concern due to its persistence in healthcare environments and its high level of resistance to antifungal agents, particularly azoles. One key mechanism underlying azole resistance is the overexpression of *CDR1*, an ATP-binding cassette (ABC) transporter gene. Gain-of-function mutations in *TAC1b*, which encodes a zinc cluster transcription factor, are also implicated in mediating azole resistance. However, the regulatory relationship between *TAC1b* and *CDR1* remains poorly understood. We recently developed an *in vitro* minichromosome isolation technique in *Saccharomyces cerevisiae* to study proteins associated with specific promoters and successfully demonstrated its utility in identifying regulators of *PDR5*, a homolog of *CDR1*. In this study, we aimed to adapt this technique to investigate transcription factors associated with the *C. auris CDR1* promoter.

**Materials & Methods:**

We successfully developled in vitro minichromosome isolation combined with proteomic analysis to identify transcription factors binding to the *CDR1* promoter in C. auris as follows. Frist, two clade I strains—CDC-0387 (azole-susceptible) and CDC-0390 (azole-resistant)—were transformed with DNA cassettes expressing LacI-V5. Whole-cell lysates from transformed C. auris were then incubated with a plasmid containing the *CDR1* promoter and eight lactose operator repeats (8×*lacO*). LacI-V5 binds to those *lacO* sites, and proteins in the lysate assemble on the plasmid, forming minichromosomes *in vitro*. Minichromosomes were then immunoprecipiated using anti-V5 antibody, and proteins on minichromosomes were finally identified by LC-MS/MS. Candidate transcription factors were further investigated by generating gene deletion mutants using the CRISPR-Cas9 system. The impact on gene expression was evaluated by RT-qPCR and RNA-seq.

**Results**:

Seven transcription factors—**Tac1a, Tac1b, Mrr1-like (B9J08\_005155), Cwt1 (B9J08\_005107), Zcf11 (B9J08\_000348), Fgr15 (B9J08\_001289)**,and **Mig1 (B9J08\_002219)—**were identified as binding to the *CDR1* promoter. Deletion mutants were successfully generated for ***TAC1a, TAC1b, MRR1-L,*** and ***CWT1***. Interestingly, deletion of ***TAC1b*** from CDC-0387 led to a significant increase in ***CDR1*** expression, while deletion of ***TAC1b-A640V*** allele from CDC-0390 led to decreased ***CDR1*** expression. These findings suggest that Tac1b can function as both a repressor and an activator of *CDR1* expression in C. auris. Tac1a and Cwt1 exhibited minor regulatory effects on *CDR1* expression, while Mrr1-like had no discernible impact on it. Furthermore, deletion of *TAC1b-A640V* caused significant downregulation of expression of ***MDR1***, a gene encoding a major facilitator superfamily (MFS) transporter.

**Conclusions**:

Our study demonstrates that in vitro minichromosome isolation is an effective method for identifying transcription factors binding to the ***CDR1*** promoter in C. auris. We identified key regulators, particularly **Tac1b**, whose dual regulatory role in *CDR1* expression offers new insights into the transcriptional control of azole resistance. Further studies are warranted to elucidate the broader regulatory network and functional mechanisms underlying antifungal resistance in C. auris.