Summary

FRG1 has garnered significant research interest owing to its increased expression levels which are associated with the pathophysiology of Facioscapulohumeral Muscular Dystrophy (FSHD). Research over a decade revealed that its reduced expression levels exert tumorigenic effects across cancer types including- oral, gastric, prostate, colorectal, and breast cancer. FRG1 expression perturbation leads to alteration of p38-MAPK signaling and GMCSF/MEK-ERK axis in prostate and breast cancer cells, respectively, leading to cancer progression. FRG1 is also known to regulate angiogenesis in HUVECs via FGF2. All these functions make FRG1 a double-edged sword. Hence, elucidating the precise role of FRG1 in cellular functionality is crucial. Understanding its exact function will provide insights into the mechanistic aspects of diseases and set the groundwork for the development of therapeutic anticancer or anti-FSHD drugs.

Owing to its subcellular localization and association with the spliceosomal C complex, few studies indicate its potential role in regulating the RNA biogenesis machinery. Some reports either by employing in-silico databases or solely taking muscle cells as model system, tried to unravel the modulators contributing to the pathophysiology of FRG1. These studies offer biases in our understanding. To date, no comprehensive, systematic study exists that provides direct evidence regarding the upstream modulators that precisely control FRG1 gene expression. Although the involvement of FRG1 in diverse physiological processes has been reported, at present research done on FRG1 is insufficient to draw definitive conclusions regarding its single basic function, or distinct, independent functions and the underlying mechanism.

We conducted this study to investigate two aspects involving FRG1. First, to identify and determine the upstream regulators of FRG1 responsible for regulating its expression and

tumorigenic properties. Second, to explore its basic function and mechanistically explore its effect on the downstream regulatory network.

For the first part, we carried out a dual luciferase reporter assay in five different overlapping promoter fragments of FRG1 (upto 1000 bp upstream of TSS). We observed varying FRG1 transcription induction in promoter regions. In-silico analysis revealed the binding of Sp1 and DUX4 on the promoter region of FRG1 showing enhanced transcriptional activity. Conversely, the promoter fragment of FRG1 that displayed reduced transcriptional activity contained YY1 binding sequence. We confirmed the binding of the three transcription factors using site-directed mutagenesis assay. While mutating Sp1 and DUX4 binding sites led to reduced FRG1 activity, an increase in the transcriptional activity of FRG1 was observed upon mutating YY1 binding site. Protein-DNA interaction-based assays provided direct evidence of the binding of Sp1, YY1, and DUX4 on FRG1 promoter region. Our findings also suggest the interaction among transcription factors in regulating FRG1 gene expression. Altering the expression levels of Sp1, YY1, and DUX4 in various combinations resulted in parallel changes in FRG1 expression. Cell-based assays, provided additional compelling evidence supporting the critical role of these transcription factors in regulating the tumor-suppressive activity of FRG1. Furthermore, the effect of upstream transcription factors perturbation on FRG1-mediated breast tumor growth was assessed using xenograft mouse model. Injecting MCF7 cells having Sp1 and DUX4 expression resulted in decreased tumor volume and weight. However in these conditions, when FRG1 levels were depleted, we found an increased tumor volume and weight. An opposite trend was observed upon injecting MCF7 cells having YY1 knockdown that resulted in reduced tumor volume and weight. An amelioration in the tumor volume and weight was noticed upon depleting FRG1 levels. All these data provide valuable insights into the collective influence of the effect of Sp1, YY1, and DUX4 in regulating the expression and tumorigenic property of FRG1. The interplay between

these transcription factors and FRG1 may provide scope for developing therapeutic strategies, especially in breast cancer patients.

In the second part of our study, we have elucidated the basic function of FRG1. Here, we have identified genes and pathways altered upon FRG1 expression perturbation. Genome-wide expression profiling of HEK293T and MCF7 cell lines with depleted FRG1 levels revealed significant alterations in genes involved in mRNA biogenesis (NMD) and its related (spliceosome and RNA transport) pathways. We observed positive correlation of NMD genes with FRG1 in a tissue-specific manner. This was corroborated by qRT-PCR results that showed reduced expression levels of NMD genes upon FRG1 depletion. However, this reduction was found to be more profound in MCF7 cell line as compared to HEK293T cell line. Multiple sequence alignment and *in-silico* motif based discovery analysis using MEME Suite resulted in the identification of conserved stretches of nucleotide sequences with CTGGG motif on the promoter region of NMD genes. Protein-DNA binding assays (EMSA and ChIP) followed by luciferase reporter and site-directed mutagenesis assays verified the binding of FRG1 to a 5 bp sequence. All of these data suggested that FRG1 acts as a transcriptional regulator of NMD genes.

Sensing the potential of FRG1 as a regulator of NMD, delving deeper into molecular intricacy, we assessed the effect of FRG1 expression perturbation on NMD efficacy. We found an increase in NMD efficiency upon FRG1 expression reduction. Taking note of the fact that FRG1 is a structural component of spliceosome C complex, we then determined the effect of FRG1 expression perturbation on splicing efficiency. Our investigation revealed that the absence of FRG1 did not affect the splicing efficacy of transcripts while ectopic expression of FRG1 results in missplicing. Upon establishing that FRG1 inversely affects the NMD pathway, while positively regulating the expression of NMD genes, we proceeded to investigate its mechanistic aspect. We ascertained that DUX4 (upstream regulator of FRG1) does not directly

affect NMD, rather FRG1 does. We have also determined that FRG1 negatively regulates UPF1 both transcriptionally as well as translationally. Furthermore, through ubiquitin-mediated proteasomal degradation assays, we have demonstrated that UPF1 degradation is not directly regulated by DUX4, rather it is mediated via FRG1. Structurally, we have determined FRG1 to be an integral component of the spliceosome-EJC-NMD complex. We have validated our *in vitro* findings using *in vivo* transgenic FRG1 knockout *Danio rerio* model system. Consistent with our *in vitro* findings, we observed enhanced NMD efficiency in 24 hpf (hours post fertilization) and 48 hpf zebrafish embryos that were microinjected at 1-cell stage with NMD sensor construct. We did not find any change in the luciferase reporter activity in 24 hpf and 48 hpf zebrafish embryos microinjected at 1-cell stage with splicing reporter construct. All these findings clearly showed FRG1-mediated dual layered regulation of Nonsense-mediated decay pathway via ubiquitination of UPF1.

In conclusion, for the first time, our study systematically elucidated the upstream regulator of FRG1 and verified their intricate interplay in regulating FRG1's expression and tumorigenic property. The findings of this thesis also demonstrate that FRG1 expression perturbation positively affects the expression of NMD pathway genes, but downregulates the expression level of UPF1. We have identified FRG1 as a transcriptional regulator of NMD pathway genes through its binding to a novel 5 bp sequence, 'CTGGG' located in their promoter regions. Further, we confirmed FRG1's role in modulating NMD and splicing processes, identifying its unexplored basic role and the underlying molecular mechanism.