We all start life as a single cell. The process of developing from a single cell to an organism requires the precise coordination of tens of thousands of coding genes in space and time. This complex process gives rise to a myriad of cell types. Surprisingly, this coordinated gene regulation is dictated by distal-regulatory elements called enhancers. Mutations in enhancers are associated with pathologies. Enhancers locate their cognate promoter in the dense 3D space of the nucleus and deliver transcriptional machinery to promoters for their activation. They act as a single unit (singleton enhancer) or in a group (clustered enhancers) to activate target gene. Additionally, active enhancers transcribe enhancer-RNAs (eRNAs) whose functions are not know. The prevailing model suggests that an enhancer's search for a promoter is restricted within a local chromatin domain known as a Topologically Associating Domain (TAD).

My lab aims to unravel transcriptional mechanisms employed by these enhancers to regulate rate of gene expression. My lab uses cutting-edge tools such as genome-wide NGS-based assays at bulk and single cell level to sophisticated high-resolution microscopy, to unravel the functions of non-coding genome. My lab has made outstanding progress in significantly enhancing our understanding of how enhancers function, and has challenged the pre-conceived notions in the field. Several aspects of enhancer biology have emerged from my own work during my stint as post-doctoral fellow and now from my own laboratory.

Following are 10 important studies:

1. Li W[#], **Notani D[#]**, Ma Q, Tanasa B, Nunez E, Chen AY, Merkurjev D, Zhang J, Ohgi K, Song X, Oh S, Kim H-S, Glass CK, and Rosenfeld MG*. Functional Importance of eRNAs for Estrogen-dependent Transcriptional Activation Events. **Nature**. 2013. 498(7455):516-20. **(# these authors contributed equally to this work). Recommended by Faculty of 1000 Biology.**

   Comment In: Redmond AM, Carroll JS. Enhancer-derived RNAs: 'spicing up' transcription programs. **EMBO J**. 2013. 32(15):2096-8.

   Research Highlight: Carlos A Melo, Nicolas Léveillé, and Reuven Agami. eRNAs reach the heart of transcription. **Cell Research**. 2013. doi: 10.1038/cr.2013.97.

2. Li W, **Notani D**, Rosenfeld MG. Enhancers as non-coding RNA transcription units: recent insights and future perspectives. **Nat Rev Genet.** 2016 Mar 7. doi: 10.1038/nrg.2016.4

I and another colleague, reported that, 17β-oestradiol (E2)-bound ERα causes a global increase in eRNA transcription on enhancers adjacent to E2-upregulated-coding genes. These eRNAs, as functional transcripts, exert ligand-dependent induction of target coding-genes, increasing the strength of specific enhancer-promoter looping initiated by ERα binding. Further, cohesin contributes to E2-dependent gene activation by stabilizing E2/ER-α/eRNA-induced enhancer-promoter looping.

Bidirectional eRNAs were just reported however, it was unclear whether these eRNAs were functional. This paper was the first report assigning regulatory functions to eRNAs. By virtue of binding with cohesion, eRNAs stabilize enhancer:promoter looping. Later, similar discoveries were with other lncRNAs and role of cohesin in loop-extrusion came much later.

However, these mechanisms of the enhancers are just the "tip of the iceberg" and Intrigues me to pursue these fascinating regulatory elements in my own laboratory.

3. Harismendy O[#], **Notani D[#],** Song X, Rahim NG, Tanasa B, Heintzman N, Ren B, Fu XD, Topol EJ, Rosenfeld MG, Frazer KA. 9p21 DNA variants associated with coronary artery disease impair interferon-γ signaling response. **Nature**. 2011. 470(7333):264-8. **([#] These authors contributed equally to this work). Recommended by Faculty of 1000 Biology.**

GWAS had strongly linked SNPs in the 9p21 gene-desert with coronary artery disease (CAD) and type-2-diabetes (T2D). Despite evidence for a role of the associated gene-desert in neighbouring gene regulation, the biological underpinnings of these genetic associations were not yet been explained. We identified 33 enhancers in 9p21 gene-desert and found that the CAD risk alleles rs10811656 and rs10757278 were located in one of these enhancers and disrupted a binding site for STAT1. Using a new open-ended technique 3D-DSL that I developed, we found that the enhancers physically interacted with the INK4/ARF locus. IFNg activation strongly affected these enhancers and the structure of the chromatin in the 9p21 locus.

Our findings functionally established a link between genetic susceptibility and genetic variation in gene desert regions. The study being first to uncover the importance of any gene desert regions in disease context has changed our view of non-coding genome.

4. Walavalkar K, Saravanan B, Singh AK, Jayani RS, Nair A, Farooq U, Islam Z, Soota D, Mann R, Shivaprasad PV, Freedman ML, Sabarinathan R, Haiman CA, **Notani D***. A rare variant of African ancestry activates 8q24 lncRNA hub by modulating cancer associated enhancer. **Nature Communications**. 2020.11(1):3598.doi: 10.1038/s41467-020-17325-y **(Editor's choice article)**

Over 3000 genome-wide association studies (GWASs) published until 2017 have identified susceptibility loci to over 1,800 unique traits and common diseases. Most of these susceptibility loci are associated with non-coding genetic variation, especially in the enhancers. Such a genetic variation can be exploited to discover mechanisms of enhancer action and in the long term develop therapeutic approaches that are driven by such enhanceropathies.

Towards this goal, genetic variation at the 8q24 locus is linked with the greater susceptibility to prostate cancer in men of African ancestry and one such African ancestry specific rare variant, rs72725854 (A>G/T) (~6% allele frequency) has been associated with a ~2-fold increase in prostate cancer risk. However, the functional relevance of this variant was unknown. Here we show that the variant rs72725854 is present in a prostate cancer-specific enhancer at 8q24 locus. Chromatin-conformation capture and dCas9 mediated enhancer blocking establish a direct regulatory link between this enhancer and lncRNAs PCAT1, PRNCR1 and PVT1. The risk allele ('T') is associated with higher expression of PCAT1, PVT1 and c-myc in prostate tumors. Further, enhancer with the risk allele gains response to androgen stimulation by recruiting the transcription factor SPDEF whereas, non-risk alleles remain non-responsive. Elevated expression of these lncRNAs and c-myc in risk allele carriers may explain their greater susceptibility to prostate cancer.

The study is the first report where a rare mutation in an enhancer has been functionally shown to lead to the susceptibility to a disease. The toolkit generated in the study can be adopted to understand the susceptibilities of the Indian population to several diseases and cancer.

5. Farooq U, Saravanan B, Islam Z, Walavalkar K, Singh AK, Jayani RS, Meel S, Sudha Swaminathan S, **Notani D***. An inter-dependent network of functional enhancers regulates transcription and EZH2 loading at INK4a/ARF locus. **Cell Reports**. 2021. 34(12):108898

6. Blobel GA*, Higgs DR*, Mitchell JA*, **Notani D***, Young RA*. Testing the super-enhancer concept. **Nat Rev Genet**. 2021;22(12):749-755. doi: 10.1038/s41576-021-00398-w.

The conventional thinking is that super enhancers regulate target gene as a sum of all enhancers. However, these observations are pure speculation but not based on functional studies. WE tested these assumptions by functionally dissecting one of the most dense-enhancer clusters in the genome at the 9p21 locus which is a GWAS hotspot. We observe that not all enhancers within a cluster are functional

and that functional enhancers form an interdependent network where they rely on each other for a net effect on target gene transcription. Furthermore, not all functional enhancers are biochemically marked with high levels of enhancer marks. The study has challenged current computational based assumptions and has prompted the field to revisit these predictive concepts again.

7. Islam Z, Saravanan B, Walavalkar K, Farooq U, Singh AK, Radhakrishnan S, Thakur J, Pandit A, Henikoff S, **Notani D***. Active enhancers strengthen insulation by RNA-mediated CTCF binding at chromatin domain boundaries. **Genome Research**. 2023 Jan;33(1):1-17. doi: 10.1101/gr.276643.122.

While dissecting the hierarchy of enhancers within the clusters at the 9p21 locus, we discovered that functional enhancers directly assist TAD boundaries to recruit CTCF (a transcription factor present at most TAD boundaries and its interactions with cohesin complex organizes TADs). Briefly, as the enhancers become active during signaling/development, they loop with boundaries of the same TAD. This looping results in the transcription of non-coding RNAs at the boundaries. These RNAs then interact with CTCF to stabilize its binding at the TAD borders. As a result, these TADs are better insulated and do not allow the enhancers to interact with genes outside the given TAD. The study has shown the novel non-canonical functions of enhancers in genome organization.

8. Saravanan B, Soota D, Islam Z, Majumdar S, Mann R, Meel S, Farooq U, Walavalkar K, Gayen S, Singh AK, Hannenhalli S, **Notani D***. Ligand dependent gene regulation by transient ERα clustered enhancers. **PLoS Genet.** 2020 Jan 6;16(1):e1008516.

9. Mann R, **Notani D***. Transcription factor condensates and signaling driven transcription. **Nucleus**. 2023 Dec;14(1):2205758. doi: 10.1080/19491034.2023.2205758.

Early on in my career, I was able to uncover the roles of eRNAs in estrogen dependent enhancer:promoter looping and gene regulation. This work laid the groundwork for several new projects and since then, eRNAs have been a subject of great interest in the field of transcription and have been shown to play various roles apart from signalling and even in development. When I started my independent laboratory at NCBS, my group began with asking how transcriptional response to cyclic hormonal signaling is so reproducible (estrogen cycles during menstruation and glucocorticoids peaks every 24h). We hypothesised that since estrogen responsive enhancers regulate gene transcription, these enhancers could carry a memory (bookmarking) between each cycle of ligand stimulation ensuring faithful signaling response.

Interrogating enhancer memory, my group recently showed that the ERα binding in the genome is clustered where one lead enhancer in the cluster is marked (pre-seeded) by an unliganded receptor even before signaling. These clusters emerge upon signaling and though they disappear at the end of signaling, they leave behind the same pre-marked enhancer still bound by the receptor for next round of ligand exposure. These enhancer clusters are identified as phase separated ERα-condensates and any perturbation in pre-seeding abolishes the enhancer-cluster formation and therefore, the transcription of genes and also their periodicity. We are now testing the extent of enhancer bookmarking in other signalling systems including hormones such as androgens and glucocorticoids. Overall, our study is the first to show that basal signaling wires the genome to respond to signaling cues in a manner that involves fast, reproducible and specific transcriptional response.

10. Soota D, Saravanan B, Mann R, Kharbanda T, **Notani D***. RNA binding limits the ligand induced transcriptional potential of estrogen receptor-alpha (ERα).
bioRxiv 2023.08.10.552751

Transcription factors (TFs) are recruited on DNA predominantly via their DNA-binding domain (DBD). Many TFs also interact with RNA and the role of RNA in TF binding and transcription regulation is

poorly understood. Understanding the influence of TF:RNA interactions on gene regulation is crucial, given that the RNA-binding domains in TFs are frequently mutated in cancers.
Estrogen receptor-alpha (ERα) is one of the earliest examples of TFs shown to interact with RNA. In this manuscript, we observed that ERα utilizes its RNA binding ability to interact with weak ERE motifs (ERα binding motifs). Lack of RNA binding causes weaker but dynamic association of ERα with chromatin. Unexpectedly, the dynamic association results in higher polymerase loading on chromatin, leading to robust transcription of a subset of ERα regulated genes upon estrogen stimulation. Taken together, with complementary genetic, biochemical, imaging and molecular studies, this manuscript: (a) provides a rich data set of ERα target RNAs, genomic targets and nascent RNA that are dependent on RNA binding ability of ERα; (b) points to a novel essential function of RNA in limiting the transcriptional potential of ERα.

In summary, our work is comprehensive and by doing so, we uncovered the novel function of RNA as transcriptional impediment for ligand induced transcription by ERα. Our data suggest that transcription factors are trapped by RNA which does not allow their free movement limiting polymerase loading and therefore transcription output of a signaling event. These mechanistic insights are completely novel, not shown before for any TF.