

# Sequence Determinants of Nrf2 Regulatory Output

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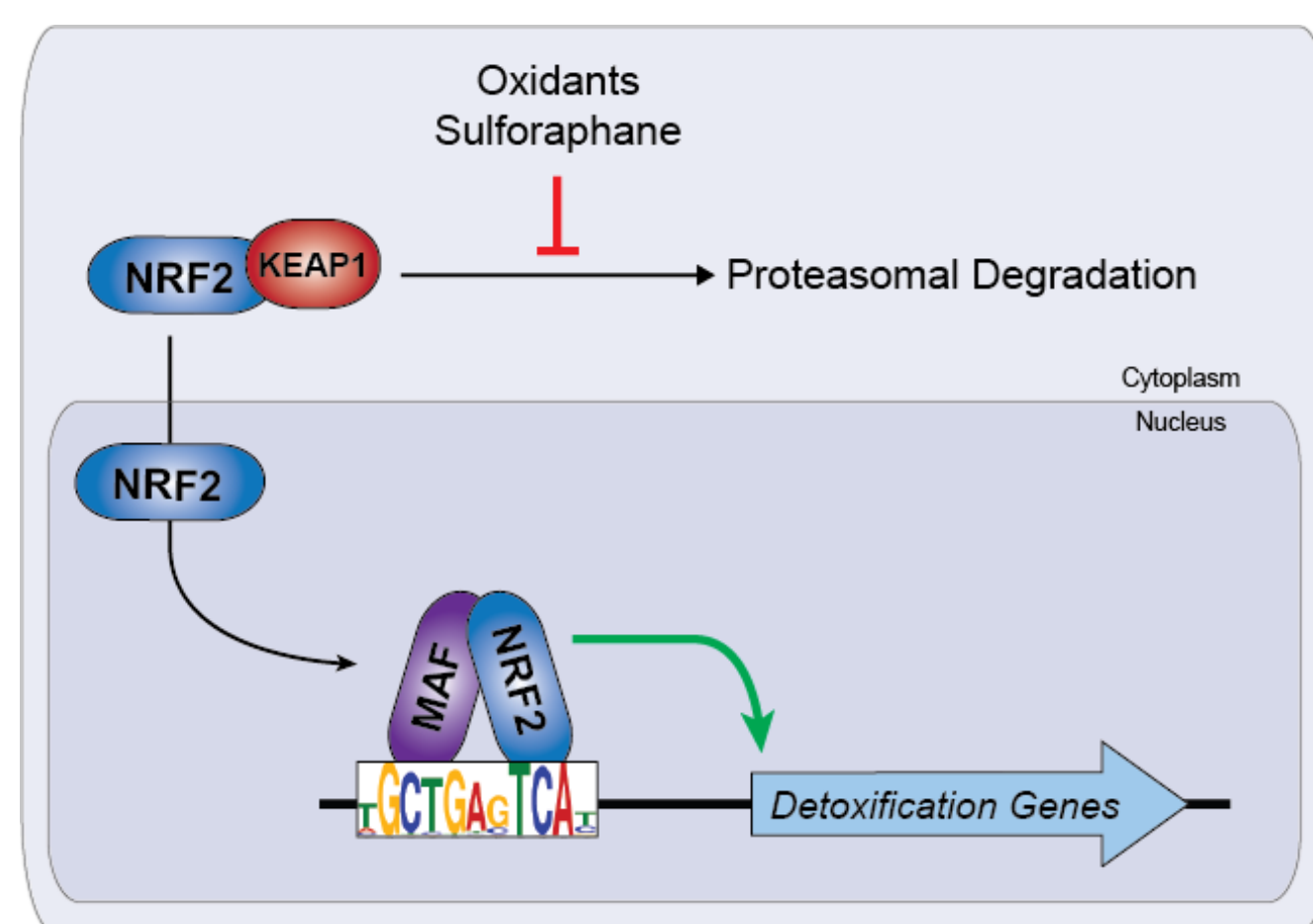
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## Introduction

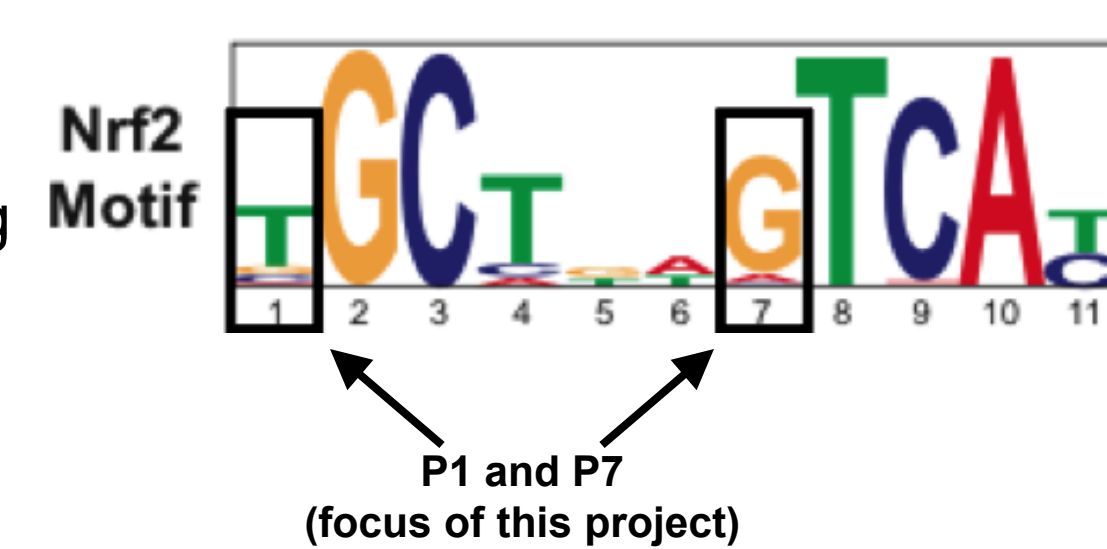


Oxidative stress damages proteins, lipids, and DNA and thus contributes to diseases such as cancer and neurodegenerative disease.

The transcription factor Nrf2 is a master regulator of the response to oxidative stress. In the presence of stress, Nrf2 translocates to the nucleus and binds a consensus DNA sequence (the antioxidant response element, or ARE) to upregulate detoxification genes.

We found that Nrf2 target genes are differentially responsive to Nrf2 activity: the differences between these responses is correlated with ARE motif quality. Some Nrf2 target genes are regulated by a perfect ARE (TGCTGAGTCAT; strong binding), while most others contain various combinations of mismatches in the variable ARE positions (weaker binding).

My recent work in the Slattery lab suggests that not all AREs are equivalent. Perfect AREs respond strongly to small increases in Nrf2 and are switch-like in responding to stress; imperfect AREs respond to Nrf2 activity in a linear manner. This data suggested that subtle changes to the ARE sequence can have a significant impact on Nrf2 binding and the corresponding regulatory output of gene expression.

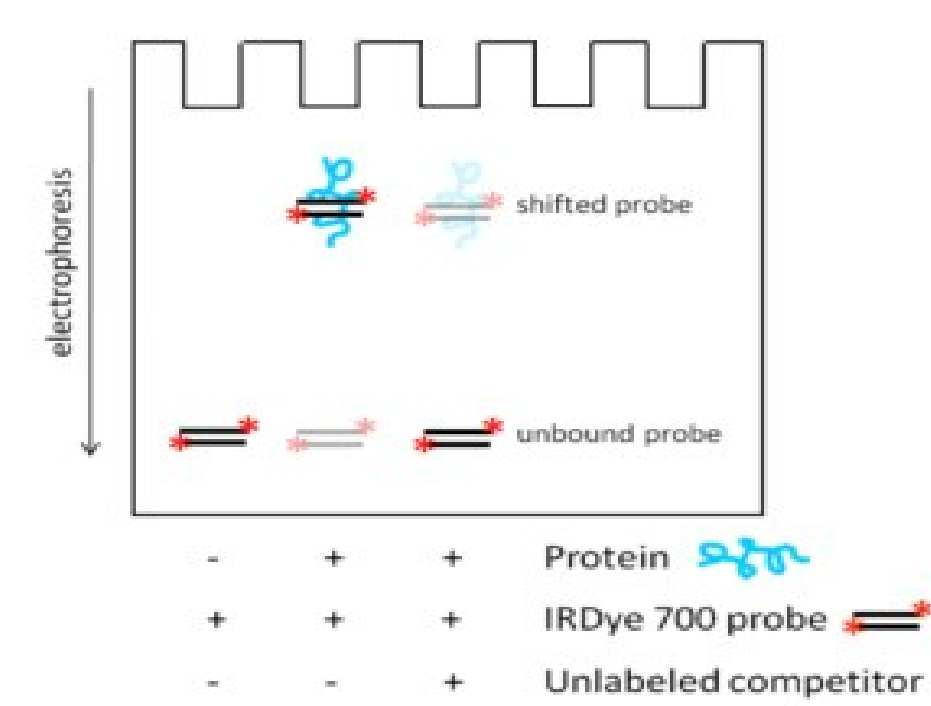


## Methods

We used luciferase reporter assays and electro-mobility shift assays (EMSA) to test the effects of seven ARE variants. Positions 1 and 7 of the ARE were varied to obtain more information on how binding motif quality influences regulatory output.

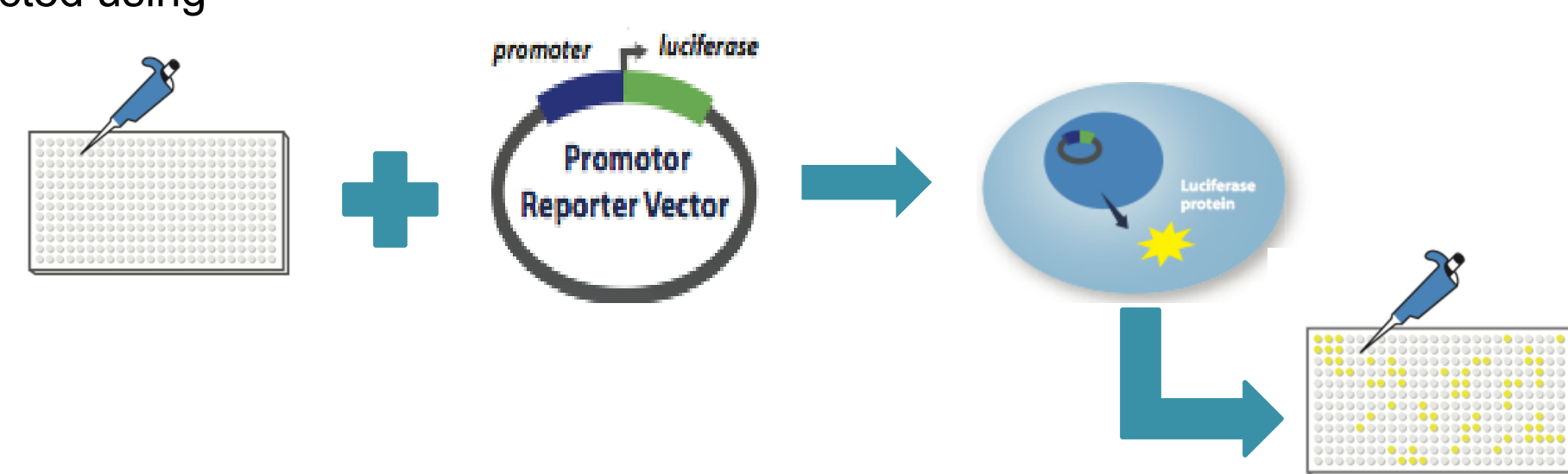
### In Vitro Methods:

• Binding of Nrf2 to the various AREs was measured using LICOR-adapted EMSA. Purified tagged Nrf2, MafG (protein), dsDNA with various AREs, binding buffer and competitor oligonucleotides were incubated and combined with orange loading dye. The solutions were electrophoresed through an acrylamide gel in 1x TBE buffer and imaged using the Odyssey LiCOR machine.



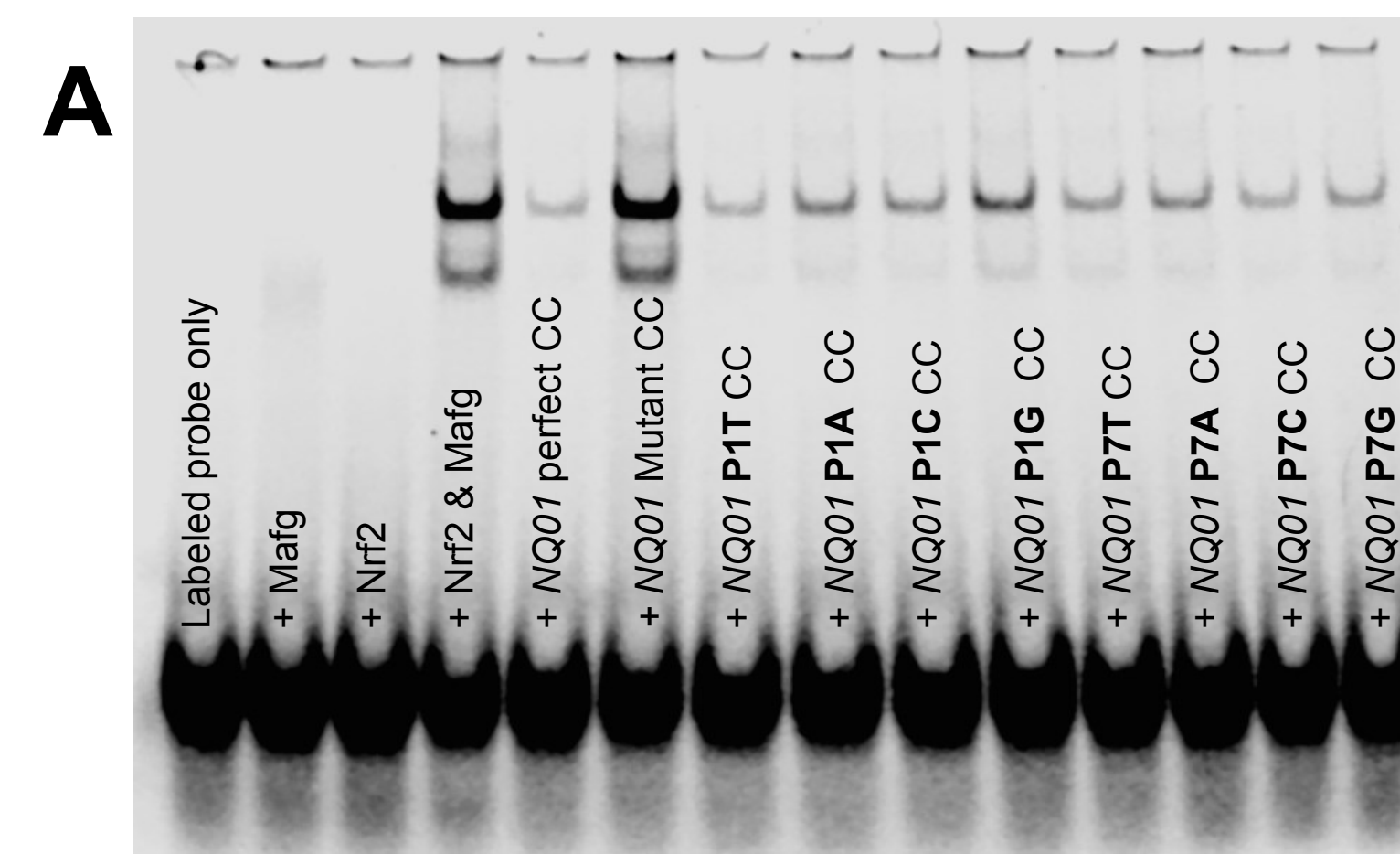
### In Vivo Methods:

• The various versions of the NQ01 ARE utilized were cloned into the LightSwitch optimized luciferase reporter vector system. All reporter assays were performed in human IMR32 cell lines. For transfection experiments, cells were seeded at 15,000 cells/well and transfected using the SwitchGear Genomic High-throughput transfection protocol. Cells were treated with sulforaphane (SFN) which activates Nrf2. Each sample was run in triplicate.

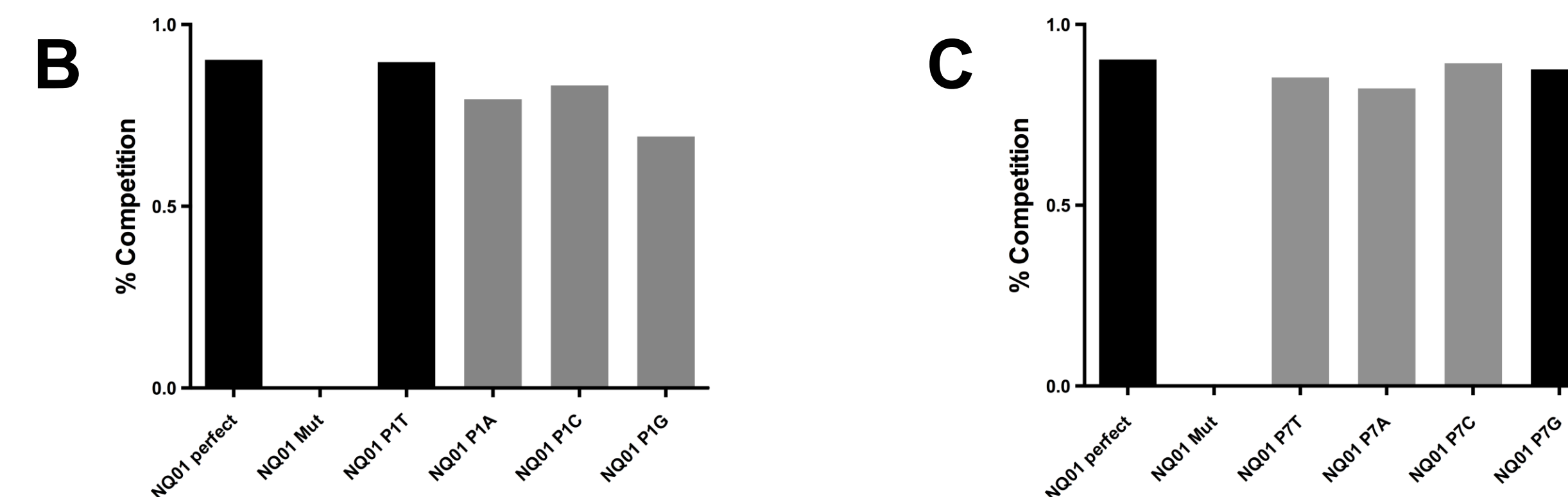


<https://www.licor.com/bio/applications/emsal>  
<http://sgg.worldzoo.net/switchgear-3/ut-goclone-reporter-assays>

## Single Nucleotide Variation in P1 and P7 Alters TF binding: In Vitro Electro Mobility Shift Assays



**Figure 1A:** Transcription factor binding to the IR-labeled NQ01 probe was seen only when both proteins, Nrf2 and Mafg, were present. Perfect and positional variant versions of the ARE found in NQ01 were used as control cold competitors (CC 50x) as indicated. **Figures 1B, 1C:** EMSA lanes were quantified using ImageStudio™ imaging software and % competition was calculated relative to no CC for both P1 and P7.



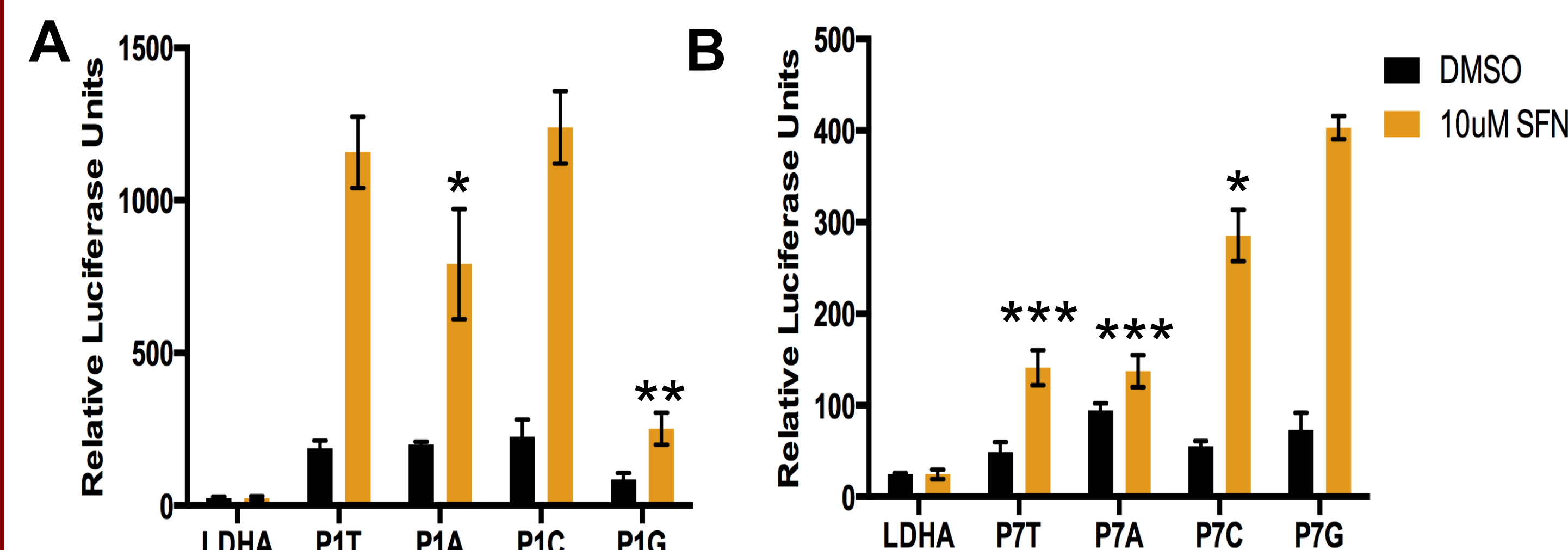
- Competition varied depending on the positional substitutions of the ARE.
- Positional variation in P1 and P7 altered Nrf2 binding to different degrees depending on the substitution.

## Single Nucleotide Variation in P1 and P7 Alters TF binding: In Vivo Luciferase Reporter Assays

**Figure 2:** The enhancer region containing the various AREs were cloned upstream of the luciferase reporter gene and evaluated in IMR32 cells. The ARE containing enhancers displayed increased luciferase activity than did LDHA control. All ARE containing enhancers were more responsive following SFN treatment than vehicle treatment.  $p \leq 0.05^*$ ,  $p \leq 0.001^{**}$ ,  $p \leq 0.0001^{***}$

**Figure 2A:** Changing position 1 from a T (perfect) to an A, C, or G altered regulatory output; T to a C substitution resulted in no change, T to a G substitution resulted in drastic change.

**Figure 2B:** Changing position 7 of the ARE from a G (perfect) to an A, C, or T significantly altered regulatory output in all cases; G and C were highly responsive to the Nrf2 activator SFN. A or T bases at position 7, both of which result in less regulatory output in gene expression translates to a weaker interaction of Nrf2:ARE.



- Variation in position 1 had an impact on regulatory output when T (perfect) was altered to an A or G; variation from T to C resulted in no difference.
- All variation in position 7 impacted regulatory output.

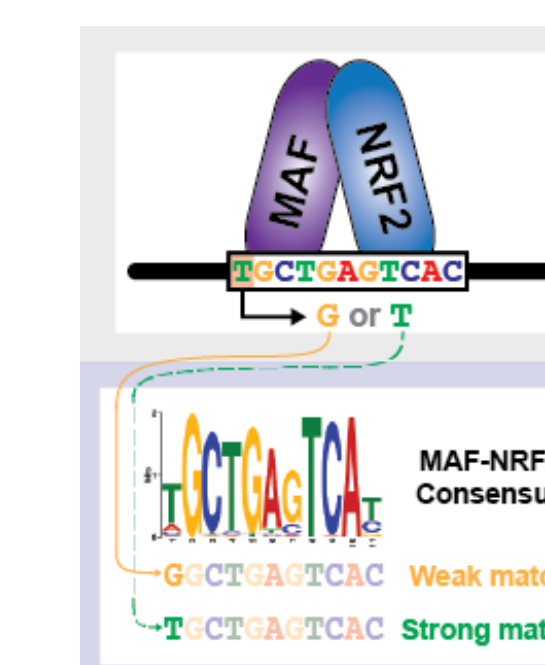
## Conclusion & Future Directions

**ARE motif quality has an impact on both Nrf2 binding and Nrf2-mediated transcriptional activation.**

Direct binding of Nrf2 to the ARE was measured *in vitro* via EMSA (Figure 1).

- Changing position 1 from a perfect T to a G had the most obvious decrease in Nrf2:ARE binding while changing T to a C had no change.
- Position 7 variation showed slightly different levels of binding.

*In vivo* Luciferase reporter assays measured how positional changes in ARE affect Nrf2 activated gene transcription (Figure 2).



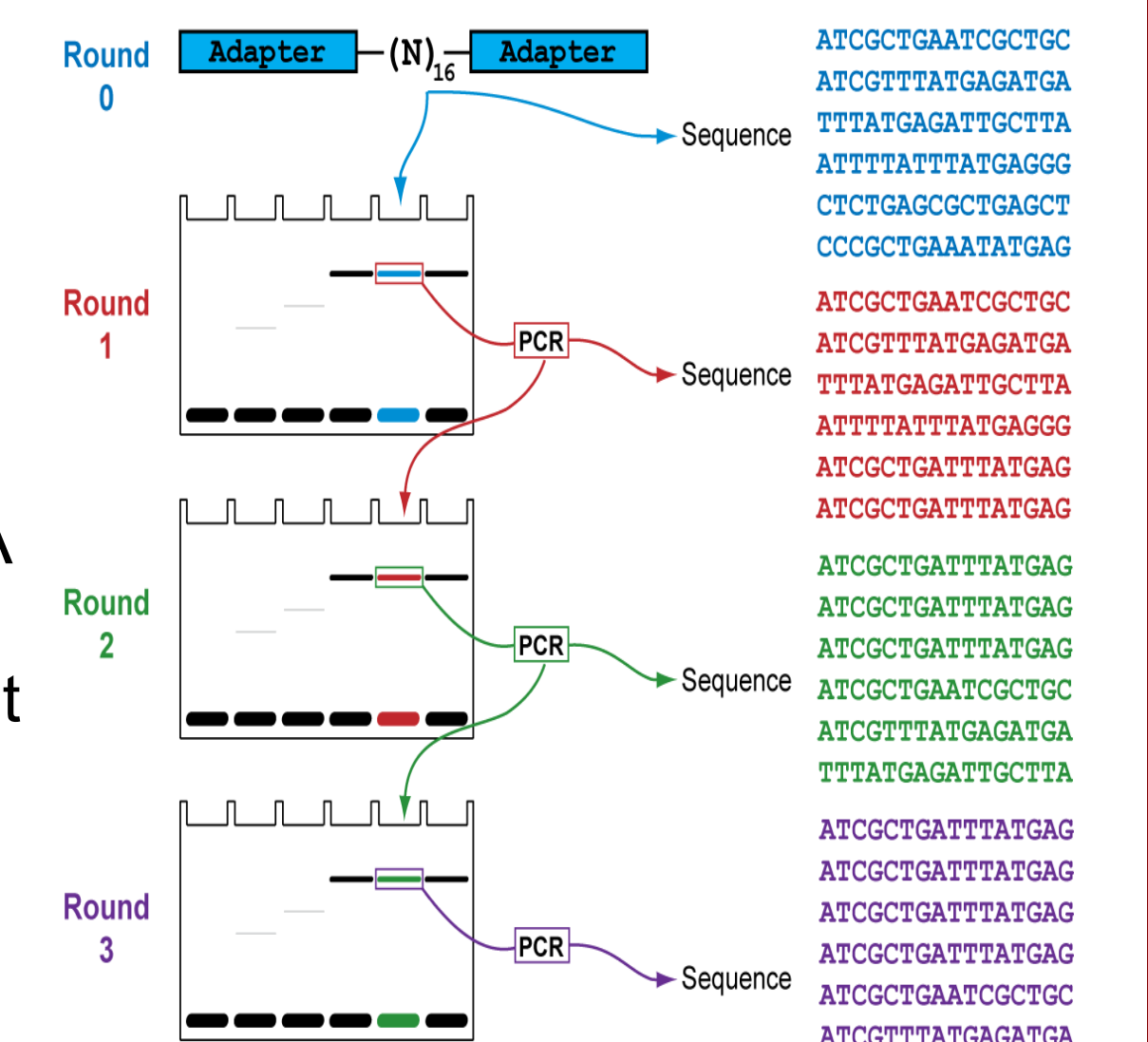
- Changing position 1 from a T (perfect) to a C resulted in no changes in luciferase expression, where as an A or G resulted in a drastic decrease of luciferase expression.
- Changing position 7 of the ARE from a G (perfect) to an A, C, or T did significantly decrease luciferase expression in all cases.

**The rules governing Nrf2:ARE interactions remain unclear.**

- Do interdependencies between positions exist (e.g., G in position 7 is only preferred when position 6 is A)?
- We need methods that will lead to a comprehensive understanding of how the positional variations in ARE binding motif translates to Nrf2 binding and gene expression changes.

**Currently I am using systematic evolution of ligands by exponential enrichment coupled with DNA sequencing (SELEX-seq).**

- DNA library containing a randomized region ( $n^{16}$  possibilities- all potential ARE combinations represented 100 times) that is flanked by defined regions is used to bind the TF interest.
- DNA bound by the complex is then separated from unbound DNA using EMSA and the bound DNA is then amplified by PCR, sequenced, and used for subsequent rounds of DNA binding and selection.
- Comprehensive view of Nrf2:ARE binding preferences.



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