DEVELOPMENT OF A HIGH-THROUGHPUT SCREENING TO IDENTIFY NFIX-MODULATING DRUGS AS NOVEL THERAPY FOR MUSCULAR DYSTROPHY

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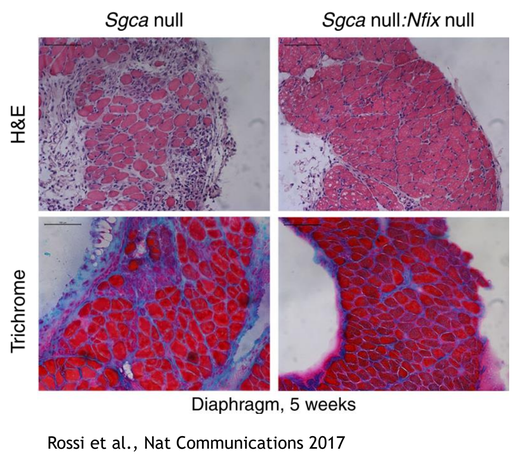
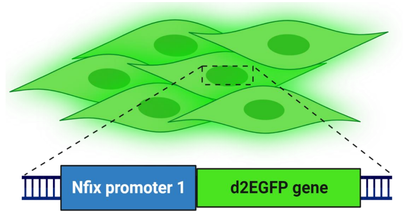
«KNOW YOUR ENEMY»: HOW TO INHIBIT NFIX IN MUSCULAR DYSTROPHIES?

Nuclear factor I X (Nfix) is a transcription factor belonging to the NFI family. Our lab demonstrated that Nfix has a harmful role in Muscular Dystrophies (MDs): indeed, its genetic deletion in skeletal muscles and macrophages leads to both morphological and functional improvements of murine dystrophic muscles (Rossi et al. 2016, 2017), thanks to a delay of degeneration-regeneration cycles, a switch towards more oxidative myofibers and a reduction of fibrosis (Saclier et al. 2022). Therefore, targeting Nfix might be a cutting-edge approach for a novel treatment of MDs. Among our different strategies to inhibit Nfix in a dystrophic context, we planned to develop a **High-Throughput Screening (HTS)** assay on a cell-based system combined with a specific bioinformatic analysis to identify possible compounds that might inhibit the *Nfix* gene expression.

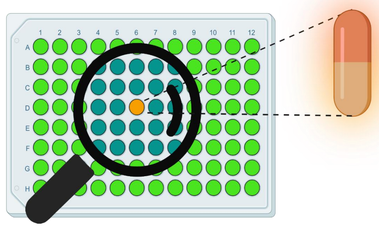


Rossi et al., Nat Communications 2017

CELL-BASED SYSTEM



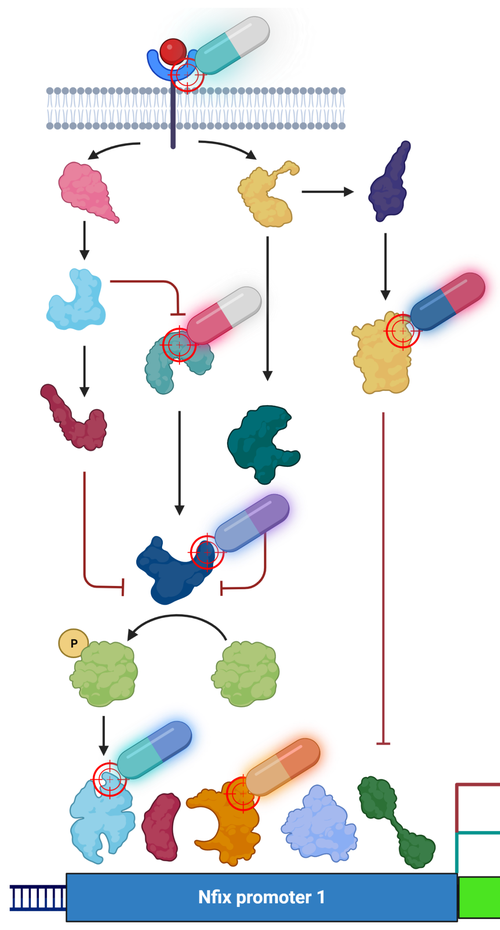
HIGH-THROUGHPUT SCREENING



DRUG LIBRARY

An efficient HTS assay requires two fundamental features: a proper **cell-based system** with a distinct phenotype and a **drug library** from which selecting the best compound able to induce the phenotypic perturbation in the cell-based. To achieve that, we set up a fluorescent myogenic cell-based system thanks to a destabilized variant of the Green Fluorescent Protein (d2EGFP) whose expression is under the control of the *Nfix* promoter 1 (pNfix1). Moreover, we designed a custom drug library made of 88 compounds which specifically might affect the *Nfix* and *d2EGFP* gene expression.

DRUG LIBRARY: CUSTOM LIST OF 88 SPECIFIC COMPOUNDS



3) Collection of **88 drugs** that might, **directly or indirectly**, modulate the activity of the Nfix promoter 1

2) Detection of **signaling pathways** in which the Myogenic Transcription Factors are involved

1) Identification of **Myogenic Transcription Factors** binding the **Nfix promoter 1**

Fig 3. Pipeline to obtain the custom library of 88 Nfix-modulating drugs. We analyzed the sequence of the Nfix promoter 1 by the Eukaryotic Promoter Database (EPD), identifying a list of transcription factors (TFs) that might bind it. Then, we merged this list of TFs with an RNA-seq analysis of wild-type and Nfix-null myoblasts, applying proper bioinformatic filters and collecting only the myogenic TFs. Therefore, we investigated in which molecular pathways and biological processes the transcription factors are involved by the DAVID Gene Ontology Database and the KEGG Pathway Database. Thereafter, searching on the DrugBankOnline Database and the Therapeutic Target Database, we collected a list of 88 key compounds that directly target the myogenic TFs or indirectly the upstream modulators in the signaling pathways previously investigated.

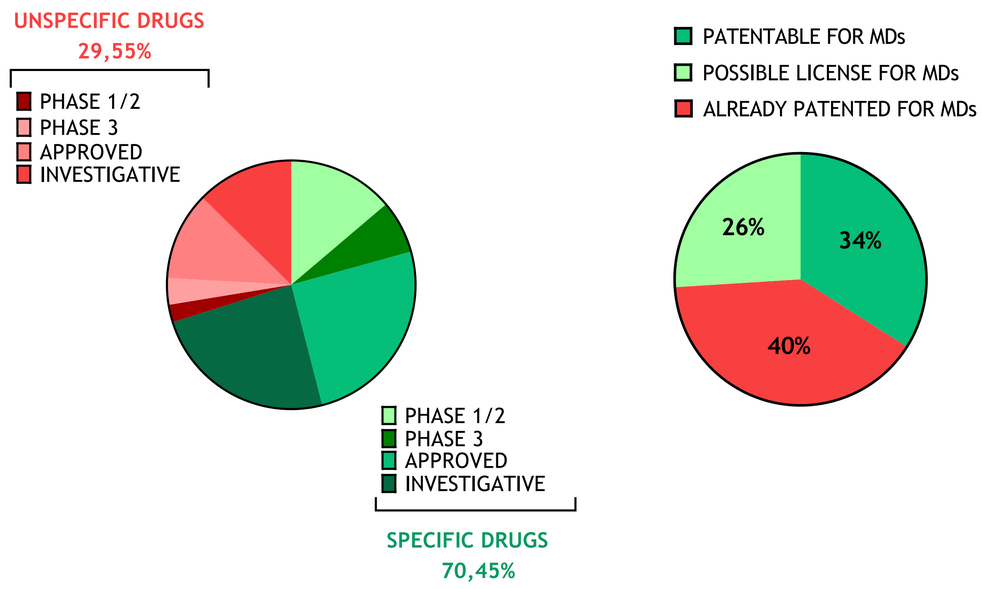


Fig 4. Specificity and patentability of the 88 drugs in our custom library. The graph on the left indicates how many drugs in our custom library are specific or unspecific for the selected TF, divided for their phase in clinical trial (phase 1/2, phase 3, approved, and investigative). The graph on the right depicts the patentability of the selected 88 drugs for MDs. Three out of five of our drugs might be patent or repurpose for treatment of MDs; 40% of them were already patented for MDs, suggesting that our unbiased analysis has conducted with high quality.

CELL-BASED SYSTEM: NFIX-RELATED FLUORESCENCE IN MYOGENIC CELLS

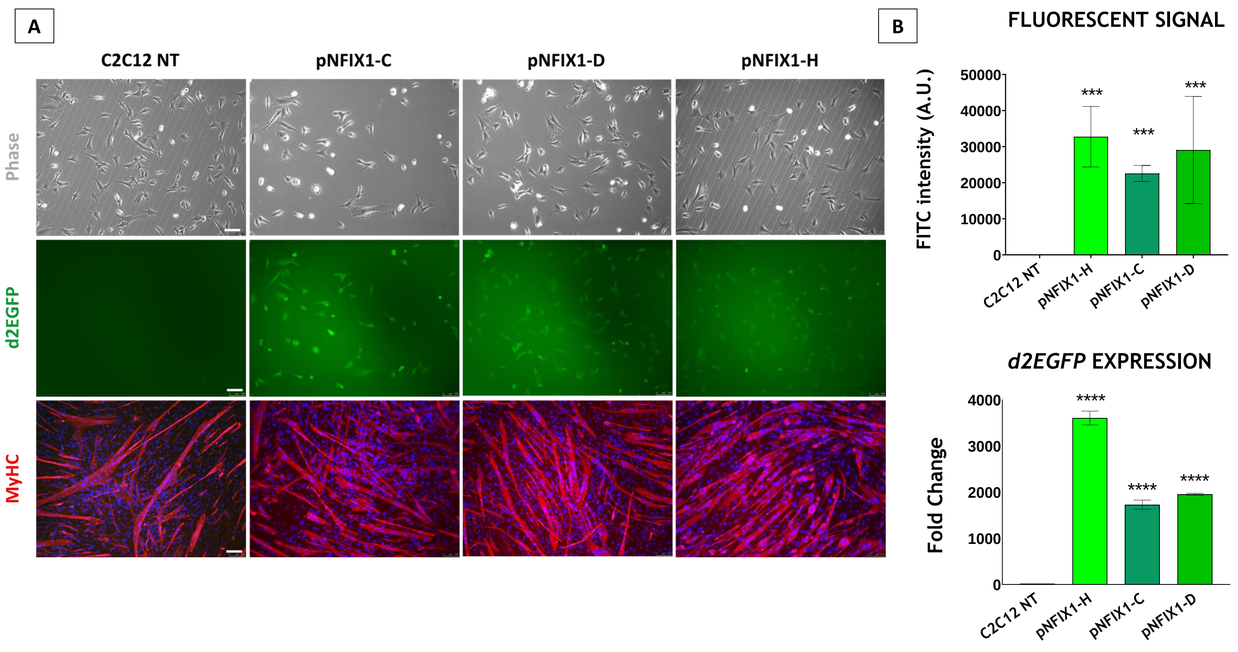


Fig 1. Fluorescent signal and myogenic behavior of the pNFIX1-d2EGFP clones. (A) In the top and middle lines, fluorescent signals from proliferating pNFIX1-d2EGFP clones (pNFIX1-C, -D, -H) and C2C12 not transduced (C2C12 NT). In the bottom line, immunofluorescent staining of Myosin Heavy Chain (MyHC) to evaluate the myogenic differentiation of the pNFIX1-d2EGFP clones compared to the control (C2C12 NT) after 6 days in differentiation medium (White Bar = 100 nm).

(B) Graphs depicting the total fluorescent signals by expression by ImageXPress Micro Confocal (top graph) and the d2EGFP gene by qRT-PCR in C2C12 NT and pNFIX1-d2EGFP clones (Ordinary One-Way Anova Test ***: p-value < 0.001; ****: p-value < 0.0001; n=3)

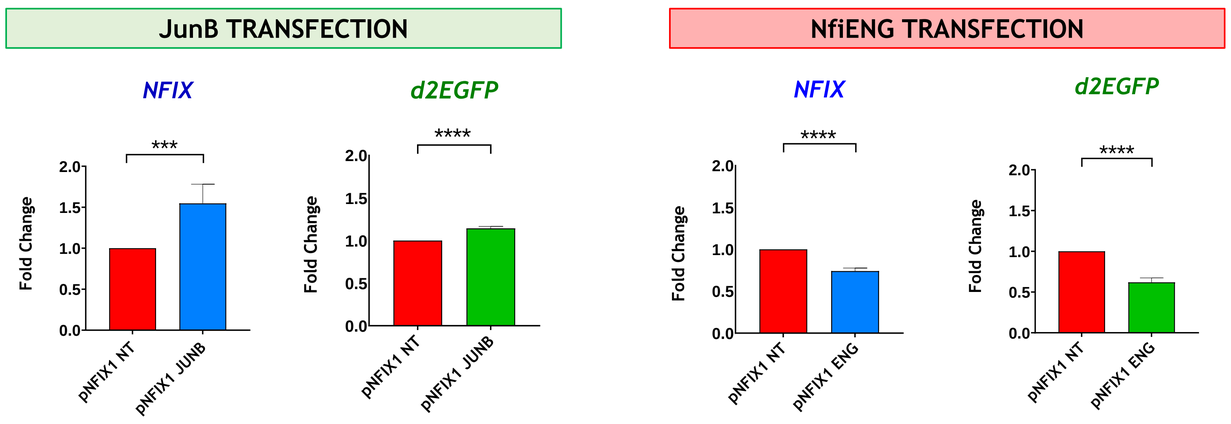


Fig 2. Responsivity of pNFIX1-d2EGFP clones to changes in the *Nfix* gene expression by JunB or NfiENG. Graphs depicting the Fold Change values of the *Nfix* and *d2EGFP* gene expression after transfection of JunB **(left)** or NfiEng **(right)** in pNFIX1-d2EGFP clones (pNFIX1 JUNB or pNIFX1 ENG) and not transfected cells (pNFIX1 NT) as control. JunB is a known positive modulator of the *Nfix* gene (Taglietti et al. 2018) while NfiENG inhibits the expression of the *Nfix* gene (Messina et al. 2010) (Unpaired t-test, ***: p-value < 0.001; ****: p-value < 0.0001; n=3; *ß-actin* was used as house-keeping gene)

CONCLUSIONS

➢ Cell-based system:

- ✓ Proper **fluorescent signal** related to the **d2EGFP** expression;
- ✓ Mainteinment of **myogenic behavior**;
- ✓ **Responsivity** to Nfix modulating-stimuli.

➢ Custom drug library:

- ✓ Identification of **transcription factors** binding the Nfix promoter 1;
- ✓ Detection of **88 key compounds** that might modulate the *Nfix gene* expression

FUTURE PERSPECTIVES: IMPROVEMENT OF THE CELL-BASED SYSTEM