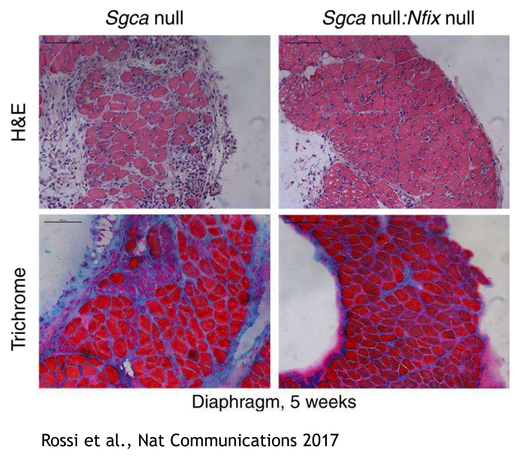
DEVELOPMENT OF A HIGH-THROUGHPUT SCREENING TO IDENTIFY NFIX-MODULATING DRUGS AS NOVEL THERAPY FOR MUSCULAR DYSTROPHY

Gabriele Rovetta[1], Lucrezia Bertazzoli[1], Giuseppe Angelini[2], Serena di Savino[3], Raffaele Epis[1], Giorgia Careccia[1], Chiara Bonfanti[1], and Graziella Messina[1]

[1]Department of Biosciences, University of Milan, Milan, Italy
[2]Institute of Functional Genomics, CNRS UMR 5203, Montpellier, France
[3]Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy

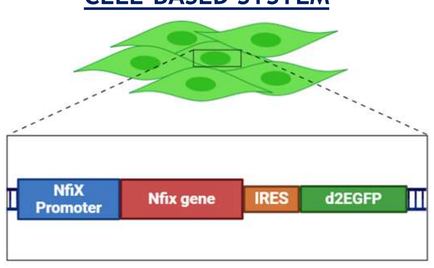
HOW TO INHIBIT NFIX IN MUSCULAR DYSTROPHIES

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). 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.

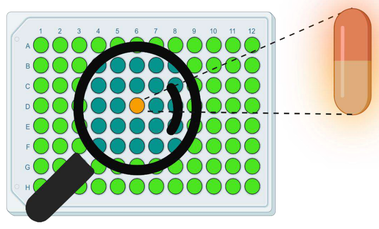


Rossi et al., Nat Communications 2017

CELL-BASED SYSTEM



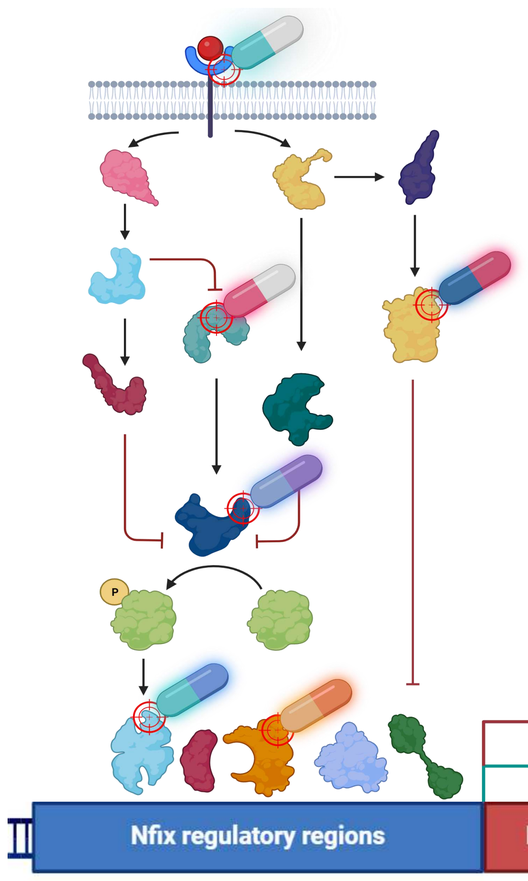
HIGH-THROUGHPUT SCREENING



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.

DRUG LIBRARY: CUSTOM LIST OF 98 SPECIFIC COMPOUNDS



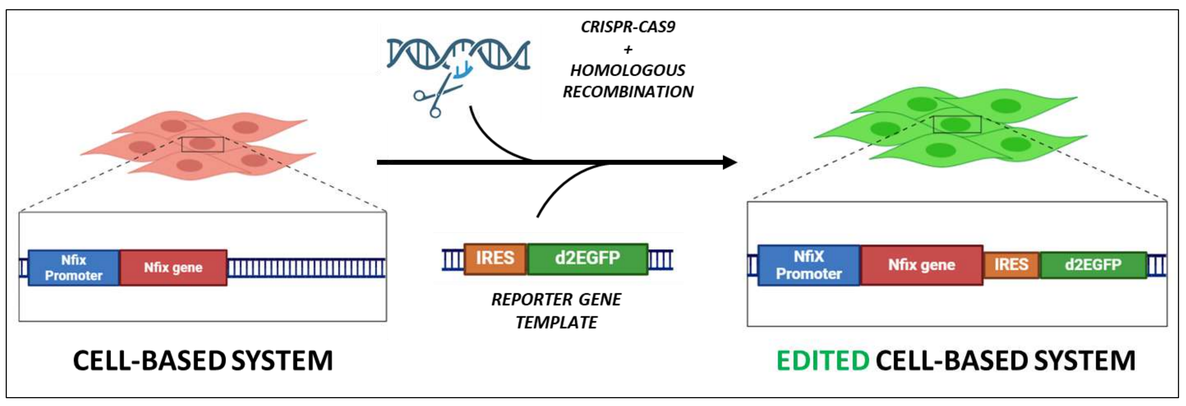
3) MCE MedChemExpress, TTD, DRUGBANK online — Collection of **98 compounds** that might modulate the ***Nfix* gene expression**

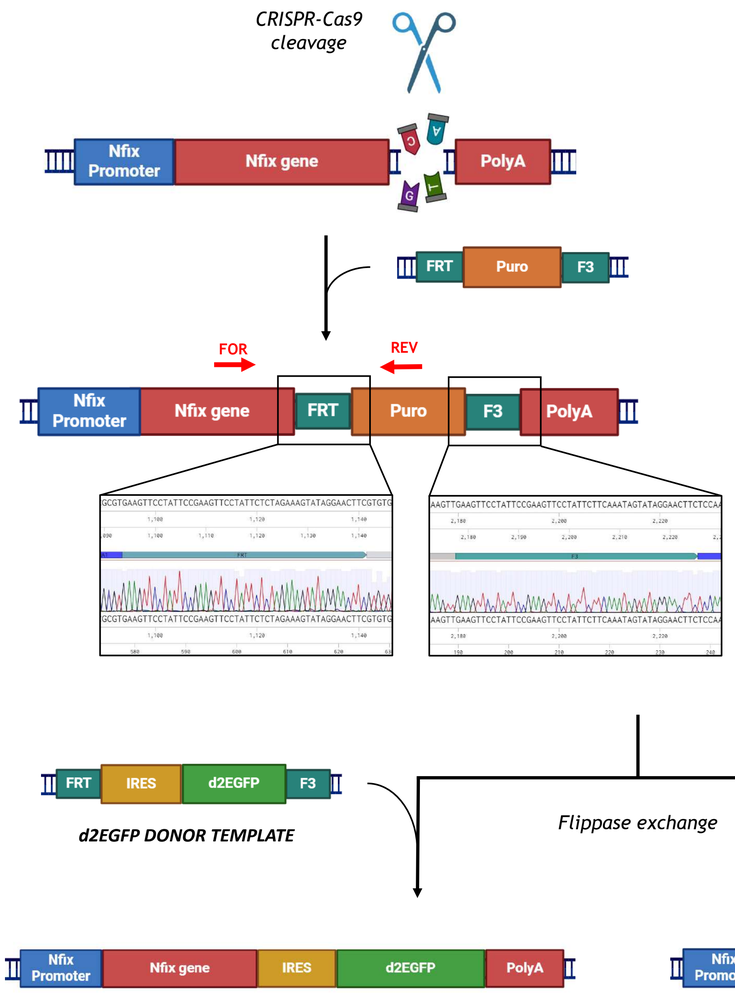
2) DAVID, RNA-seq, KEGG — Detection **of Myogenic Transcription Factors** and their signaling pathways

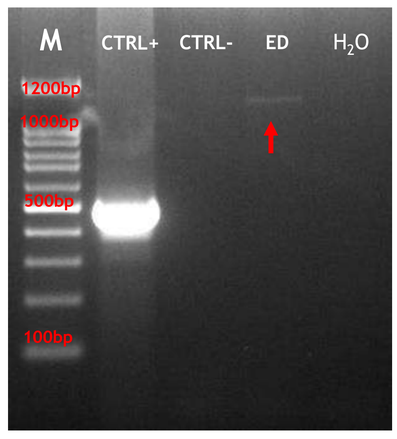
1) UCSC, JASPAR2024, SIB EPD Eukaryotic Promoter Database — Identification of **Transcription Factors** binding to the **Nfix regulatory regions**

Fig 3. Pipeline to obtain the custom library of 98 Nfix-modulating compounds. We analyzed the sequence of the Nfix regulatory regions using Eukaryotic Promoter Database (EPD) and UCSC, identifying a list of transcription factors (TFs) that might bind it from JASPAR. Then, we merged this list of TFs with an RNA-seq analysis of wild-type and Nfix-null myotubes, applying proper bioinformatic filters and collecting only the myogenic TFs. Thereafter, searching on the DrugBankOnline Database, MedChem Express website, and the Therapeutic Target Database, we collected a list of 98 key compounds that directly target the myogenic TFs or indirectly the upstream modulators in the signaling pathways previously investigated.

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







ACADEMIC APPLICATIONS

COMPANY APPLICATIONS

Fig. 1 Pipeline of the protocol used to generate the reporter myoblast cell line. In the left it is represented the protocol developed to generate d2EGFP- or Luciferase- Nfix myoblast cell line dependent by the *Nfix* gene expression: At first, we promoted CRISPR-Cas9 cleavage between the end of the Coding Sequence and the PolyA signal. Then, we integrated the FRT-PURO-F3 cassette and we generated an adaptable cell-based system useful to create a d2EGFP- or Luciferase- myoblast cell line related to the Nfix expression. To the right, it is depicted the agarose gel confirming the presence of the FRT-PURO-F3 cassette at the desire locus (CTRL+: plasmid sequence, CTRL-: not edited myoblasts, ED: edited myoblasts).

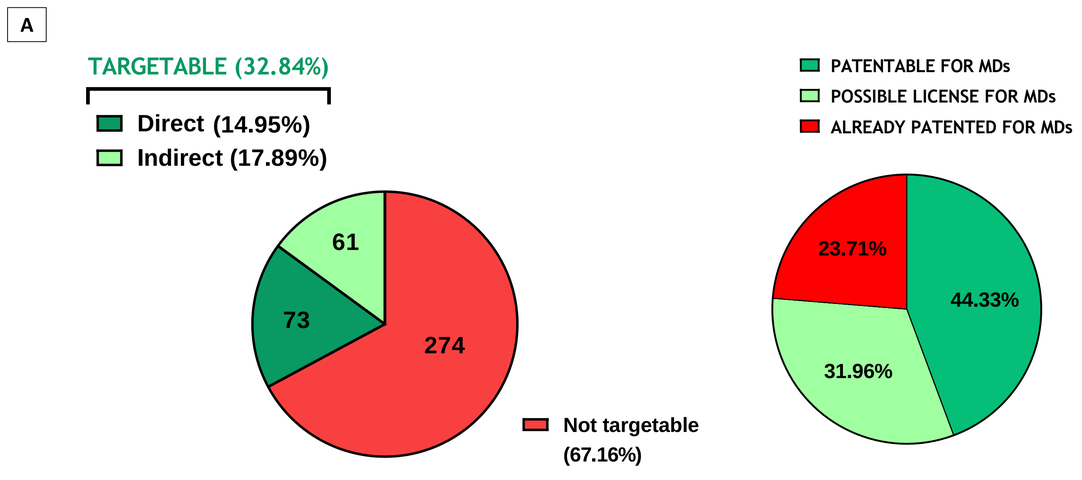


Fig 4. Targetability and patentability of the 98 drugs in our drug library (A). Actvity of compound T-5224 on the *Nfix* gene expression (B). (A) The graph on the left indicates how many TFs are targetable, while the graph on the right depicts the patentability of the selected 98 drugs for MDs. **(B)** To the left, illustration of the action of compound T-5224 (25uM) on *Socs3* and *Nfix* gene. To the right, qPCR results of *Socs3* and *Nfix* gene expression obtained after T-5224 treatment on murine myoblasts (Relative Gene Expression on DMSO). The treatment was performed adding the compound daily for 3 days at 25 uM (n=2).

CONCLUSIONS

➢ Cell-based system:

Generation of efficient **editing protocol** to obtain adaptable heterozygous puromycin resistant myoblast cell line:

- **Editing efficiency: 62%**
- **Editing specificity: 98%**

➢ Custom drug library:

- ✓ Identification of myogenic **transcription factors** binding the Nfix regulatory regions and their pathways ;
- ✓ Detection of **98 key compounds** that might modulate the *Nfix gene* expression
- ✓ Analysis of **T-5224** as promising **Nfix inhibitor**

FUTURE PERSPECTIVES: IMPROVEMENT OF THE CELL-BASED SYSTEM

➢ Production of murine and human d2EGFP- or Luciferase- Nfix cell based system

➢ Development of High-Throughput Screening assay to identify Nfix modulators

- ❑ Compounds **repressing Nfix** expression: Muscular Dystrophies, Medulloblastoma
- ❑ Compounds **promoting Nfix** expression: Esophageal Squamous Cell Carcinoma, Marshall-Smith Syndrome