High-throughput screening of the hypoxia pathway: examining promoter response and function

Johanna Samuelsson[1], Shelley Force Aldred[1], Nathan Trinklein[1], Menghang Xia[2], Ruili Huang[2] and Jim Hudson[3]

[1]Active Motif, Inc. Carlsbad, CA. [2]NIH Chemical Genomics Center, Bethesda, MD. [3]Hudson Alpha Institute for Biotechnology, Huntsville, AL.

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INTRODUCTION

Many studies have highlighted the benefit of conducting genome-wide expression and transcription factor binding studies in parallel. However, after generating these descriptive data sets, a number of questions remain. Which genomic elements are responsible for transcript level changes and what is the effect of a transcription factor binding event? Using functional reporter assays to study the behavior of genomic elements in living cells can help answer many of these questions. To enable these studies in a high-throughput format, we have created a genome-wide library of human promoters and UTRs in a luciferase-based reporter system. Our unique collection of validated assays allows you to measure gene expression changes associated with a number of disease-related biological pathways, including hypoxia.

The hypoxia pathway, mediated through the hypoxia-inducible factor 1 (HIF-1), is essential to normal growth and development and is involved in the pathophysiology of cancer, inflammation and ischemia. Hypoxic conditions in tumors also cause resistance to radiotherapy and chemotherapy, creating considerable interest in the development of anti-hypoxia drugs. Although HIF-1 inhibition in hypoxic tumor cells may confer therapeutic benefits, evaluating the full HIF-1 pathway is critical for efficient screening of small molecules and minimizing off-target effects. In this study, we assembled a panel of known and potentially novel hypoxia-related promoters to measure the response and determine the function of these promoters, including naturally occurring sequence variants, to different treatments and conditions in high-throughput.

The LightSwitch™ System for pathway studies

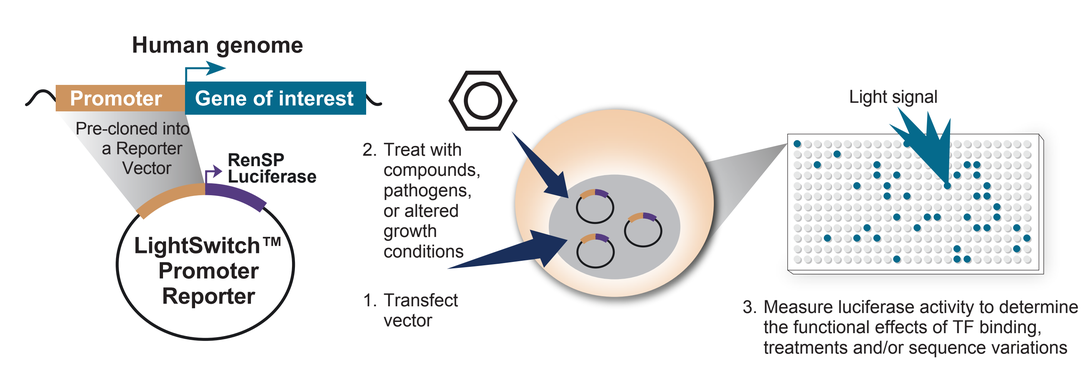


Figure 1. Experimental design for a pathway functional screen using the LightSwitch System. Cells are transcfected with an individual promoter or synthetic response element reporter vector followed by treatment with compounds or altered growth conditions at a suitable time point. Luciferase reagent is added 24-48 hours after transfection and the plate incubated for 30 minutes in the dark before being read on a luminometer. Promoter activity is calculated as the ratio of the treated cells over vehicle-only control.

Profile pathway response to treatment

DISEASE PROFILING PANEL
48 promoters and controls

Pathway activity readout for:

HIF-1α	Hypoxia
NFkB	Inflammation
CREB	cyclic AMP
HSF1	Heat shock
p53	DNA damage, apoptosis
STAT	Interferon
SREBP	Cholesterol biosynthesis
ER	Estrogen
AR	Androgen
GR	Glucocorticoid
AhR	Toxicity

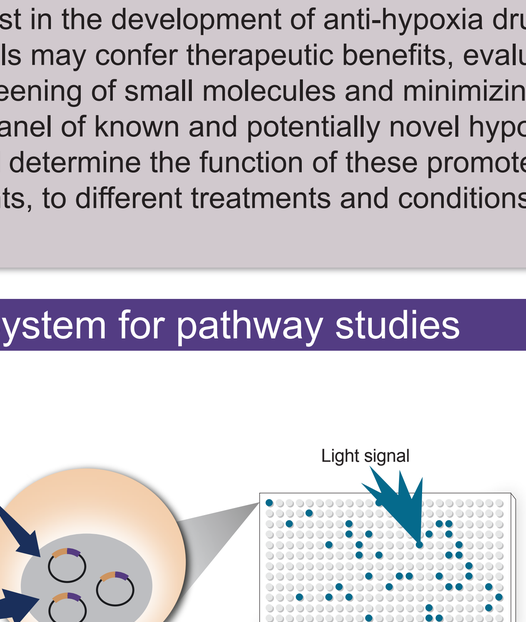


Figure 2. Screen full regulatory networks to create pathway activity profiles. This heat map shows the inducible activity of 48 different reporter constructs (rows) in 15 different conditions (columns). The red boxes indicate promoters that are up-regulated in a certain condition whereas blue boxes indicate down-regulated promoters. The conditions used in these experiments are treatments with known inducers of different biological pathways. All experiments were performed in HT1080 cells, and the 48 reporters are a combination of endogenous human promoters and synthetic response elements cloned into LightSwitch vectors.

The HIF-1 regulated hypoxia pathway

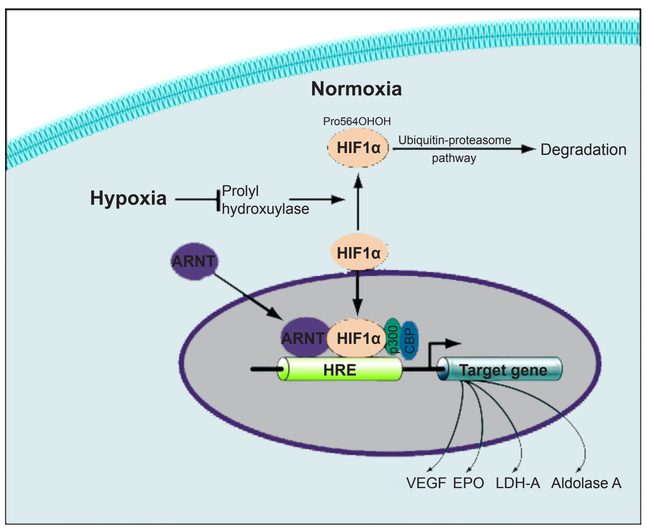


Figure 3. The HIF-1 regulated hypoxia pathway. During normoxia, HIF-1α is rapidly degraded by the proteasome pathway. However, under hypoxic conditions, prolyl hydroxylase activity is attenuated, which decreases the proteasomal degradation of intracellular HIF-1α. The accumulated HIF-1α heterodimerizes with HIF-1β and translocates into the nucleus. The HIF-1 complex binds to DNA regulatory sequences known as HREs, which are present in the promoter or enhancer regions of HIF-1 target genes. After recruiting transcriptional coactivators, such as p300 and CBP, the expression of target genes including VEGF, EPO and LDH-A, are initiated. Abbreviations: ARNT = aryl hydrocarbon receptor nuclear translocator; EPO = erythropoietin; LDH = lactate dehydrogenase.

A high-throughput hypoxia pathway screen

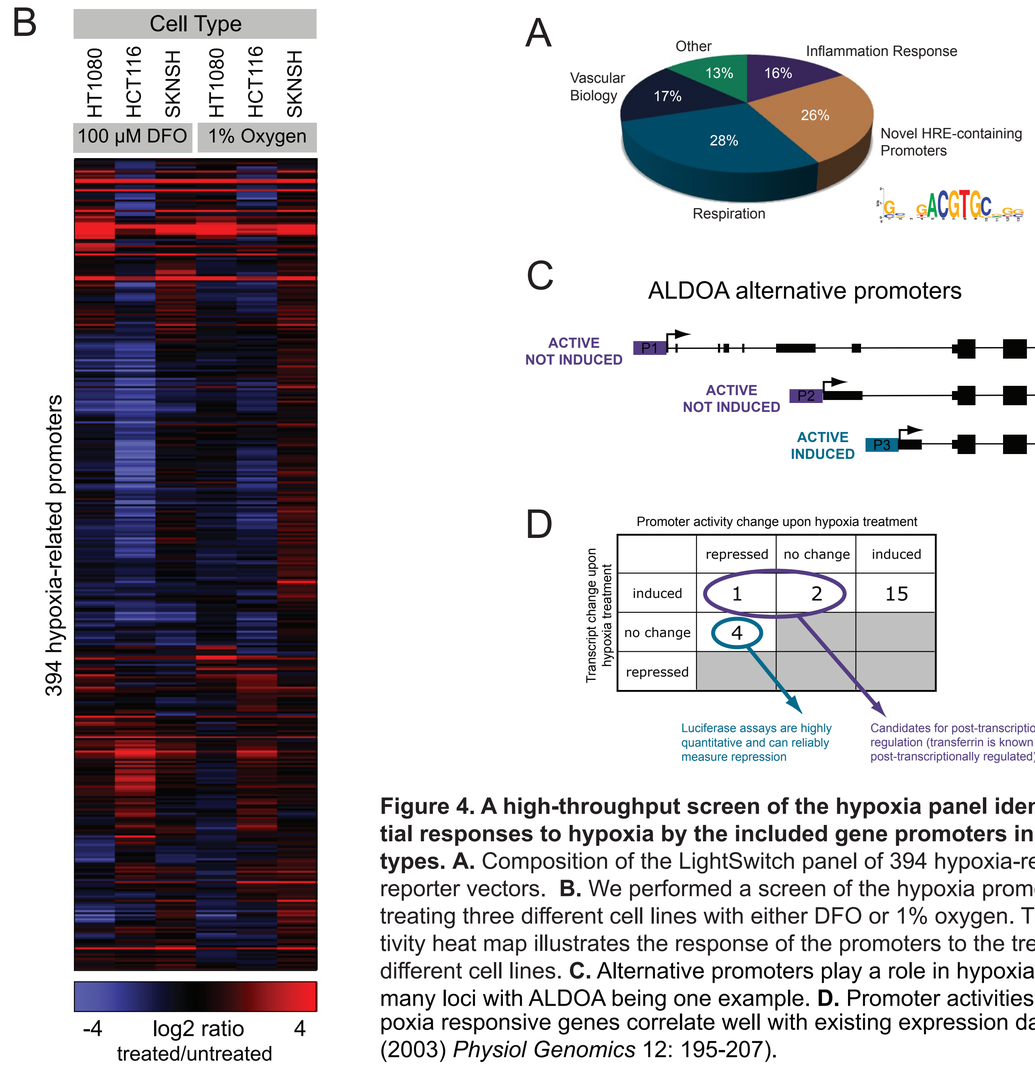


Figure 4. A high-throughput screen of the hypoxia panel identifies differential responses to hypoxia by the included gene promoters in different cell types. A. Composition of the LightSwitch panel of 394 hypoxia-related promoter reporter vectors. **B.** We performed a screen of the hypoxia promoter panel by treating three different cell lines with either DFO or 1% oxygen. The promoter activity heat map illustrates the response of the promoters to the treatments in the different cell lines. **C.** Alternative promoters play a role in hypoxia regulation for many loci with ALDOA being one example. **D.** Promoter activities for 22 known hypoxia responsive genes correlate well with existing expression data (Sonna *et al.* (2003) *Physiol Genomics* 12: 195-207).

Determine HIF-1α motif and sequence variant effects

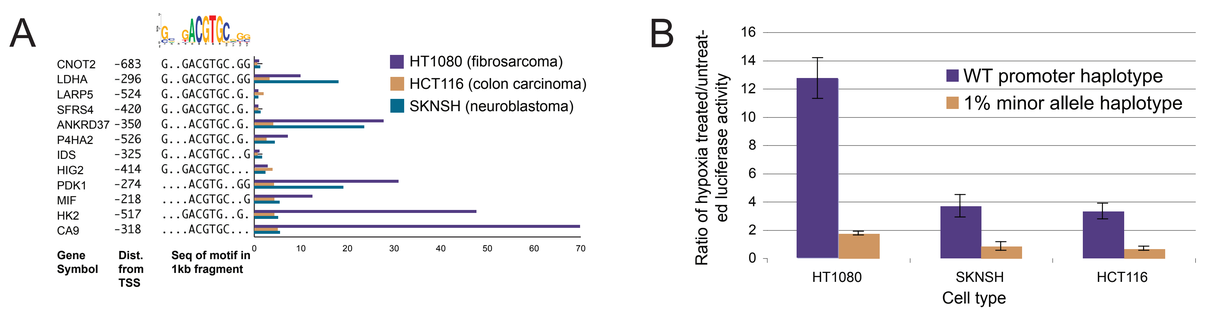
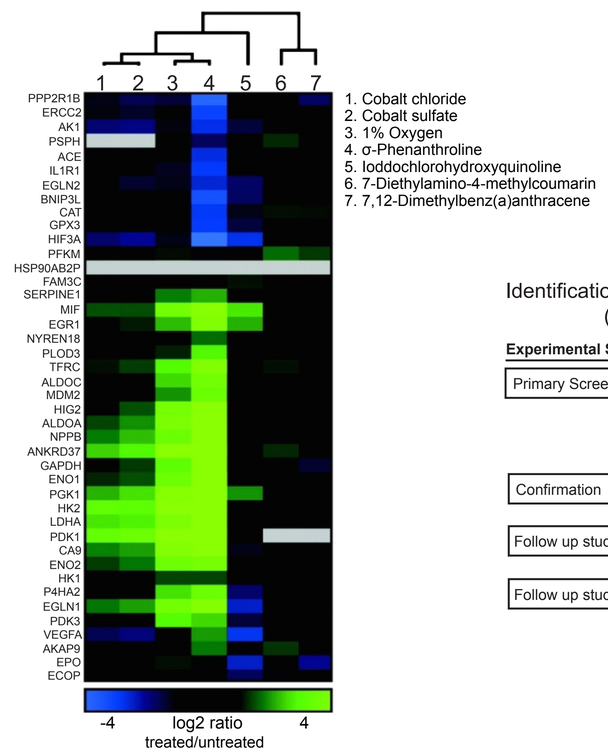


Figure 5. HIF-1α motif and sequence variant analysis shows that: A. HIF-1α motif occurence does not predict functional activity, and **B.** high-throughput reporter assays can be used to measure sequence variant effects on regulatory element function.

Toxicology screen of the hypoxia pathway



Identification of Chemical Compounds that Induce HIF-1α Activity
(Xia *et al.* (2009) *Toxicol. Sci.* 112: 153-163)

Experimental Strategy	Compounds	Assay Types
Primary Screening	NTP 1408 compounds	HRE reporter cell line
	↓ Data analysis: 15 compounds identified	
	↓ 3 compounds cytotoxic	
Confirmation	12 compunds	HRE reporter cell line
	↓ 2 compounds inactive	
Follow up study	10 compunds	Human VEGF secretion
	↓ 5 compounds inactive	
Follow up study	5 compunds	HIF1α dependent VEGF secretion, HRE promoter profiling

Figure 6. Screening a toxicology panel for HIF-1α-dependent hypoxia inducers. Table: A primary reporter screen identified 15 of 1408 compounds that activate a synthetic hypoxia response element (HRE). A secondary VEGF secretion assay in WT and HIF-1α KO MEFs showed that two of those compounds induce hypoxia independent of HIF-1α. **Heat map:** We performed a secondary functional screen of 36 human hypoxia-responsive regulatory elements as a follow up study. The activity heat map illustrates hierarchical clustering for compound activation of promoter and LRE activity for the six compounds and 1% oxygen. As identified by the VEGF secretion assay, the LightSwitch hypoxia pathway screen confirms that compounds 6 and 7 are outliers as they do not induce HRE-containing promoter activity.

CONCLUSIONS

- The LightSwitch System demonstrates optimal performance when mapping and measuring the behavior of hypoxia-responsive genomic elements
- HIF-1α motif and sequence variant experiments identified regulatory elements necessary for hypoxia response
- A high-throughput hypoxia screen allowed new insight into the regulation of the human hypoxia pathway, including the identification of several HIF-1α-dependent hypoxia inducers from a toxicology panel

Contact: samuelsson@activemotif.com