~~Modular and scalable logic computation *in vivo* with transcription factor inputs. **[Kobi: Please simplify the title. It currently would mean little to a non-specialist.]**~~

Rationally-designed logic integration of regulatory signals in mammalian cells.

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Molecular-level information ~~processing on the molecular scale[1]~~ **processing[1,2], or computing, is essential for 'smart' *in vivo*** ~~nanoscale systems. Gene~~**nanosystems. Natural molecular computing, such as messenger RNA (mRNA) synthesis regulation** ~~in the cell by certain~~ **by**

special proteins called transcription factors (TFs) ~~is an example of such processing~~[3,4], ~~and designing similar systems could create~~ may inspire engineered systems leading to the next generation of nanobiotechnological and nanomedical applications. ~~Significant progress in this direction has been made by building logic cascades[5-12] from natural and synthetic transcription factors and promoters[13-15] and, yet a method to create arbitrarily-defined gene regulatory functions is still lacking.~~ Synthetic pathways[5-15] have already implemented logical control of mRNA levels by certain TF combinations. Here we show an ~~RNA-based~~alternative approach ~~to create programmable and scalable logic~~ toward general-purpose control of mRNA and protein levels by logic integration of transcription factor ~~inputs~~input signals in mammalian cells. ~~The inputs are first 'transduced' via simple sensor promoters into specially-designed artificial microRNAs; these microRNAs then regulate protein-coding~~ The factors regulate synthetic genes coding for small regulatory RNAs – microRNAs – that in turn control mRNA of interest (*i.e.*, output ~~mRNAs)~~ via RNA interference~~, implementing a desired mapping[16] between TF inputs and the output.~~ pathway. Simple nature of these modular interactions allows in theory to implement any arbitrary logic relation between the TFs and the output[16]. We construct, test, and optimize increasingly complex ~~logic~~ circuits with up to three TF inputs, establishing a platform for *in – vivo* molecular computing. ~~**Kobi, please revise your introductory paragraph using less formal language. Terms and phrases such as transcription factor, promoters, microRNA, 'protein-coding output mRNAs', and 'logic integration of transcription factor inputs' would mean little to a non-specialist. I've made only minor changes at this stage. Please take another stab at it.**~~

Our circuits contain sensory, computational and actuation modules resembling those found in engineered control systems (Fig. 1a). In the sensory module, individual TF inputs are

'transduced' into microRNA (miR) molecules by directly regulating the expression of engineered, non-native ~~microRNA~~ miR genes[17] from simple TF-responsive promoters. In the computational module, miRs expressed from these genes down-regulate the output protein-coding transcripts via RNA interference (RNAi), thereby implementing a complex and robust logic control[16]. The output protein, a fluorescent reporter in our experiments, could also be an actuator that triggers a physiological response. ~~[Kobi, please check everywhere that miR is used consistently to refer to microRNA. I've highlighted a few instances below where they have been used interchangeable. Please be consistent. Same goes for transcription factors (TFs).]~~

The circuit utilizes the following output dependency on the ~~microRNA~~miR levels:

Output = (NOT(miR-a) AND NOT(miR-b) ...) OR (NOT(miR-c) AND NOT(miR-d)...) OR (...),

where miR-a, -b, ... target the first output-encoding transcript, c, d,... target the second, *etc*. The dependency holds because a high level of at least one output ~~(?)~~-encoding transcript is required to generate the output protein, and none of the miRs that target this transcript can be present[16]. Linking miR expression to a transcriptional activator or repressor requires the corresponding TF to be absent or present, respectively, for the ~~microRNA~~miR to be absent. In Fig. 1a, TF-A activates ~~(shown by an arrow)~~ miR-a (pointed arrow), TF-B represses ~~(shown by a~~ miR-b (blunt arrow)~~ miR-b~~, TF-D represses miR-d and TF-E activates miR-e. The overall circuit logic becomes

Output = (NOT(TF-A) AND TF-B) OR (TF-D AND NOT(TF-E)).

This approach can in principle lead to a fully-predictable regulatory program, conditional on the availability of appropriately-controlled single-input promoters. The specific form of the relation

between the inputs and the output ~~falls under the definition of a 'normal' logic function; **[any regulatory program that is well-approximated by the Boolean formalism can be recast in normal form[18]. Kobi, please revise this sentence in bold. What do you mean by 'recast in normal form'? Did you mean, '…logic function, where the usual Boolean formalism can be used'?** ~~, *i.e.*, groups of AND operators connected by OR operators, is known to be universal. Therefore, any digital molecular computation with multiple TF inputs and a single protein output can be expressed using this universal form and implemented using our circuits[18].

We experimentally tested our approach using the well-characterized TFs rtTA, Rheo and LacI-Krab as inputs. Known promoters (pTRE, pRheo and pCAGop) regulated by these TFs were used to drive the expression of ~~microRNA~~ miR genes (FF3, FF4 and FF6~~ – OK to move here?) derived from~~) whose active miR products were identical to active strands of previously-tested small interfering RNA (siRNA~~ FF3, FF4 and FF6[16] **[Kobi, this sentence implies that FF3, FF4, FF6 are siRNAs. Is this correct? Please also define siRNA on first use].**~~)[16]. These ~~microRNAs~~miRs target established sequences[16] ~~in~~fused into the three prime untranslated region~~s~~ (3'-UTRs) of the ~~messenger RNA (~~mRNA~~)~~ coding for the ZsYellow fluorescent reporter output (Fig. 1, Supplementary Text~~, Fig. 1b)~~), causing mRNA degradation and **XXX? [Kobi, please briefly state what happens when**concomitant decrease in the **miR binds to the mRNA even if it seems obvious].** ZsYellow level. Using these well-defined components allowed us to focus on the proof-of-principle of our method.

We developed three different circuits with two or three TF inputs and measured their response to all possible combinations of ~~'digital'~~ **[Kobi, please use inverted commas sparingly. Please check everywhere and revise accordingly. These should be used only to imply the word has a different meaning than the conventional one or something unusual**On and

~~**should be avoided.]** (‘On’ and ‘Off’)~~ Off input states. The experiments were performed in immortalized human embryonic kidney cells HEK293. Cells were transiently transfected with the invariant part of the circuit, that is, the output and ~~microRNA~~ miR genes (Supplementary Table 1). Depending on the desired state (~~‘On’~~On or ~~‘Off’~~Off) of a TF input in a particular measurement, we added, or withheld, the DNA plasmid that constitutively expresses this factor. Each experiment ~~using~~with a particular input combination ~~of input states (‘~~and appropriately-modified output mRNAs (circuit~~ experiment’, or ‘circuit’ – **Kobi, please use only one abbreviation. Circuit seems appropriate in this case**~~) was accompanied by a control experiment (‘control’~~control) with the same inputs but ~~without~~ with either ~~the non-sense **[Kobi, please quickly define ‘non-sense’]** or the miR targets in the input.~~an unrelated sequence replacing the correct miR target or an absent miR target in the 3’-UTR of the output mRNAs. All non-specific output changes caused by differences in transfection efficiency, non-specific binding of TFs to circuit components, off-target RNAi, and/or other factors are accounted for in the observed output change in the control experiments. Accordingly, On/Off ratios were normalized by the corresponding ratios in the controls to extract the underlying circuit ~~behavior.~~behaviour. Note that our ~~transiently transfected~~ circuits exhibit cell-to-cell variability typical of transient ~~delivery methods, which form~~ transfections, forming a Poisson distribution with an estimated mean ranging upward of 3-4 plasmid copies per cell[19]. The strongest signal (or lack thereof) comes from the cells at the high end of this distribution, where the plasmid copy number is three to four times higher than the mean and all the plasmids are likely to be co-transfected.

~~We constructed and~~ Prior to testing multi-input circuits, we calibrated their single-input ~~circuits using a constitutive~~ ‘branches’ using Cytomegalovirus (CMV) immediate-early promoter

~~to drive the~~-driven output (Supplementary Text). ~~Next, we implemented regulatory programs with two TF inputs. In~~To facilitate calibration, miR-encoding RNAs were embedded as introns between two exons coding for a fluorescent protein, such that miR expression was accompanied by the generation of a fluorescent protein serving as a miR level reporter. Following the initial adjustment of the transfection conditions, we implemented circuits with two TF inputs. The first one ~~we~~ used the activator rtTA and the repressor LacI-Krab as inputs. ~~In this circuit,~~ LacI-Krab repressed a pCAGop-driven AmCyan-FF4 ~~construct~~ while rtTA activated pTRE-driven DsRed-FF3. The level of the fluorescent protein ZsYellow is expected to be high when rtTA is ~~'Off'~~ Off and LacI-Krab is ~~'On'~~On, or using logic notation, 'ZsYellow = NOT(rtTA) AND LacI-Krab'. This circuit behaved as ~~designed~~intended (Fig. 2a, Supplementary Fig. 5). The corrected On/Off ratios with different input combinations varied from ~ 5 to ~2, with an average of ~4.2 (Supplementary Table 3). To further confirm that ~~the observed~~these changes ~~in output~~ were ~~elicited by~~ RNAi-specific, we used ~~**fluorescent**~~flow cytometry to measure miR reporters ~~**on microRNA**~~ and output levels ~~**– Kobi, please revise. What do you mean by**~~in individual cells (Fig. 2b, Supplementary Methods). The data show that increasing miR levels lead to a decrease in the output protein, after correction for non-specific effects. Consistent with this~~**? Please revise by first stating what you have done followed by** result, inspection of microscopy images shows minimal overlap between the ~~**fluorescent**~~miR reporters ~~**bit. For example, 'To confirm…, we determined**~~ and the ~~**response function of**~~output in the circuit ~~**using single-cell FACS data from circuit and control**~~ experiments ~~**whose miR have been labeled with fluorescent reporters']**~~ ~~to determine the response function of the circuit using single-cell FACS data from both circuit and control experiments (Fig. 2a, Supplementary Methods). The data show that increasing microRNA levels leads to a decrease in the output protein, after correction for~~

~~non-specific effects. Consistent with this result, visual inspection of microscopy images suggests that in the circuit experiments the microRNA reporters do not overlap with the output~~, as opposed to significant overlap in the controls.

~~One~~ Modularity is the key attribute of our design approach ~~is its modularity~~, where only a few well-defined changes are required to alter the regulatory program. To demonstrate this feature, we replaced the pCAGop promoter of the AmCyan-FF4 fusion with a pRheo promoter leading to a new program: 'ZsYellow = NOT(rtTA) AND NOT(Rheo)'. The system behaved as expected, ~~resulting in the On/Off ratio~~generating On/Off ratios ranging from 2- to 6-fold (Supplementary Fig. 6, Supplementary Table 3~~). The transfer function was also consistent with the suggested mode of operation~~) and showing a correct relation between miR and output levels (Supplementary Fig. 7).

While these data qualitatively fitted ~~our~~ expectations, the On/Off ratios of the circuits required quantitative ~~performance of the circuits, namely the On/Off ratios, required~~ improvement. Low ratios were caused by high output levels in the ~~'Off' state, or 'leakage',~~Off state (leakage) and other non-specific effects. To improve ~~the circuits' performance we tried additional~~ performance we placed the output under the control of alternative constitutive promoters ~~to drive the output gene~~, and found that the promoter EF1a significantly reduced ~~the residual output amount~~leakage in the ~~'Off'~~Off state (Supplementary Text). We attribute this improvement to a transcriptional delay ~~in~~of the output ~~protein~~ caused by an intron in the EF1a promoter as well as to weaker absolute expression from this promoter (Supplementary Fig. 9). Therefore we used EF1a-driven output in two-input circuits without changing the remaining components. For the 'ZsYellow = NOT(rtTA) AND LacI-Krab' regulatory program using the EF1a promoter ~~**[Kobi, is this referring to the bottom panel of Fig 2a, using the EF1a**~~

~~promoter? Please make clear. If necessary, call the bottom panel, fig 2b and revise accordingly]~~, the average On/Off ratio was ~27 (Fig. ~~2a~~2c, Supplementary Fig. 10). The average ratio was ~13 in the regulatory program 'ZsYellow = NOT (rtTA) AND NOT (Rheo)', a significant improvement over the CMV-driven version (Fig. ~~2b~~2d, Supplementary Fig. 11).

Next, we created a three-input circuit to implement the program 'ZsYellow = (NOT(rtTA) AND NOT(Rheo)) OR (LacI-Krab)'. In our measurements (Fig. 3, Supplementary Table 4, Supplementary Fig. 12), seven out of eight possible input combinations behaved as predicted. One expected ~~'Off'~~Off output (rtTA is ~~'On'~~On) was relatively high yet it was still 30% lower than the lowest measured ~~'On'~~On output. This result is consistent with the relatively low On/Off ratio of the pTRE-DsRed-FF3 construct observed in single- and two-input circuits (Fig. 2, Supplementary Fig. 8)**.** The average ZsYellow level, normalized to the highest measured output, was ~0.46 among the ~~'On'~~On states and ~0.14 among the ~~'Off'~~Off states, roughly as expected (0.5 and 0, respectively, because the highest output is generated by two separate transcripts). The same circuit implemented with CMV-driven output showed greatly deteriorated performance, as expected from two-input circuit comparison (Supplementary Fig. 13). Indeed, some of the ~~'Off'~~Off states are higher than some of the ~~'On'~~On states and the average ~~'On'~~On level was ~0.5 of the maximum compared to 0.3 in the ~~'Off'~~Off state.

While our circuits' primary purpose is to respond to changing TF levels~~ of transcription factor proteins~~, in some cases those proteins ~~are themselves controllable by small molecule~~can be controlled by molecular cofactors. The activators used in our experiments require cofactors (Dox for rtTA and RSL1 for Rheo) that we used at saturating concentrations. One might consider the TFs and the cofactors as separate inputs, in which case the circuit logic would include both these species. For example, the 'NOT(rtTA) AND NOT(Rheo)' program would become '(NOT(Dox)

OR NOT(rtTA)) AND (NOT(RSL1) OR NOT(Rheo))'. There are a total of 2^4 = 16 input combinations in this formula and setting some of them permanently to False (or True) allows us to operate with a part of the truth table (Supplementary Table 5). When the TFs are constitutively expressed, the circuits could be used as logic integrators of their cognate cofactors, as shown experimentally in Supplementary Fig. 14. Independent control of TF and cofactor inputs further expands the complexity of our circuits and suggests their use as ~~tunable~~tuneable gene-expression platforms or sophisticated sensors of small molecule expression profiles in cells.

Our study has demonstrated that bottom-up construction can lead to complex systems with predictable ~~behavior. [~~behaviour. At the same time we ~~**encountered context-specific effects**~~observed that the different components are not strictly modular and that non-specific background processes can lead to deviations from expectation, a well-recognized challenge facing the construction of synthetic biological networks[10,20]. ~~**– Kobi, please revise this sentence. I don't follow. How are the first and second fragments related?]**~~ ~~Decoupling~~ More efficient decoupling of the synthetic components from the endogenous processes[21] and from each other could reduce these ~~effects.~~deviations. Fluctuations may also limit scalability, and they need to be contained by proper tuning of individual interactions such that the input effect becomes efficiently saturated at the low end of its naturally-occurring distribution in the cell population. For example, RNAi knock-down could be augmented with transcriptional repression[22]. In addition, one could use auxiliary circuitry such as bistable switches[23], ~~negative and positive~~ feedback, and ultrasensitive response[24]. Resolving these challenges will enable larger circuits, as well as cascades in which the output of an upstream computation serves as an input to a downstream circuit to engineer increasingly~~-~~ complex regulatory programs.

Future applications of our systems with exogenous TFs include ~~tunable~~tuneable gene expression systems. Extending the inputs to endogenous TFs will lead to reporters and modulators of cell states, 'intelligent' therapeutic agents and new nanotechnology applications. In particular, our tools for *in vivo* molecular computation could become essential components of smart nanobiomaterials that interact with the biological environment[1]. In this context, we envision ~~a~~ distributed and non-distributed ~~mode~~modes of system operation. In the distributed mode~~, in which~~ the TF sensors are delivered to individual cells while the computational and the actuation modules are encapsulated in artificial nanovesicles supporting RNAi and protein translation[25]. The ~~microRNAs~~miRs generated by the sensors would be shuffled to the nanovesicles using naturally-secreted exosomes[26], and the encapsulated ~~modules **[Kobi, did you mean 'components' here? Please be careful with the use of modules because it can be easily confused with the computational and actuation modules described earlier]**~~components would respond by producing a desired protein that would either be secreted or delivered to specific cells via targeted endocytosis[27]. In a non-distributed mode, the entire network could be compartmentalized in channel-equipped nanocarriers[28] with cell-free extracts ~~**[Kobi, what do you mean by 'extracts'?]**~~ supporting transcription/translation/RNAi in order to detect secreted inputs such as TFs or their ligands, and generate appropriate response species.

Methods

Cell culture. Maintenance of human embryonic kidney cells (293-H) and plasmid transfection were performed as described[16]. The summary of plasmid amounts can be found in Supplementary Table 1. All transfections were performed in 12-well plates using 1 mL of growth medium per well. To increase transfection efficiency 25 μM Chloroquine (Sigma) was added 1 h prior to transfection. 3 hours after transfection the ligands RSL1 and/or Doxycycline were added

at 1 μM and 1 μg/mL final concentrations, respectively. For fluorescence-activated cell sorting (FACS) analysis, cells were either prepared as described[16] or trypsinized with 0.1 ml 0.25% trypsin-EDTA. After addition of 0.5 ml DMEM (Invitrogen) the cell suspension was collected and used directly for FACS analysis. The output was assayed 48 h post-transfection and quantified using population-based readouts (Supplementary Methods).

siRNA molecules. SiRNAs were obtained from Dharmacon and processed as described previously[16].

Recombinant DNA constructs. The details of the cloning strategy are described in Supplementary Methods. Most of the constructs were made using standard restriction-ligation methods, and the exon-intron constructs were made using seamless cloning approaches[29] with SII-type SapI restriction enzyme (~~NEB). MicroRNA~~New England Biolabs). MiR stem-loop templates were designed based on published siRNA sequences[16] following the guidelines of the pPRIME method[30].

Microscopy measurements. All microscopy images were taken from live cells grown in glass-bottom wells (Mattek) in the transfection medium supplemented with 10% FBS. We used Zeiss Axiovert 200 microscope equipped with shutter filter wheels, Prior mechanized stage and an environmental chamber (Solent) held at 37 ºC during measurements. The images were collected by an Orca ERII camera cooled to -60 ºC, in the high precision (14 bit) mode using a 20x PlanApochromat NA 0.8, PH2 objective. The collection settings for the fluorophores were S500/20x (excitation) and S535/30m (emission) filters for ZsYellow, S430/25x (excitation) and S470/30m (emission) filters for AmCyan and S565/25x (excitation) and S650/70m (emission) for DsRed. A dichroic mirror 86004v2bs (Chroma) was used for ZsYellow and AmCyan. The

dichroic mirror 86021bs (Chroma) was used for DsRed. Data collection and processing were performed by the Metamorph 7.0 software (Molecular Devices). All images illustrating a given circuit and its control underwent the same processing.

FACS measurement and data analysis. 50,000-150,000 cells from each transfected well of 3 biological replicas were analyzed on a BD LSRII flow analyzer. ZsYellow was measured using a 488 nm laser, a 505 nm longpass filter and a 530/30 emission filter. AmCyan was measured with a 405 nm laser, a 460 nm longpass filter and a 480/40 emission filter. DsRed was measured with 561 nm laser and a 585/20 emission filter. The data were compensated and analyzed as described in Supplementary Methods. To account for non-specific changes in the output, we corrected the data as follows: the mean values of the ZsYellow output for the data set obtained by flow cytometry for 'circuit' experiments were divided by the mean ZsYellow outputs for the corresponding control experiments prior to averaging over three biological replicas and normalizing by the highest measured 'On' state.

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Author contribution. Y.B. designed research and supervised the project. M. L., L. B., J. L., Z. X. and Y.B. performed research. M. L., Y.B~~.~~., J. L. and ~~J.~~ L.B. wrote the manuscript.

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Figure Legends

Figure 1 | Design elements of synthetic circuits. a, An example of a logic circuit with multiple transcription factor inputs and a fluorescent ZsYellow protein output. Three different system modules are shown. Transcription factor inputs A through F, promoters P_A through P_F, ~~microRNAs~~miRs miR-a to miR-f and output-encoding mRNA transcripts containing miR targets T_a to T_f are indicated. Pointed arrows denote activation, and blunt arrows represent repression. Elements in gray denote potential directions for circuit scale-up. **b,** Detailed structure and shorthand notation for the constructs used in this report. 'Ex' denotes exons, and other structural elements are as indicated. ~~**[Kobi, please check if a 'p' is missing from Rheo and CAGop in panel b.]**~~

Figure 2 | Experimental implementation of two-input regulatory programs. Plasmid amounts are given in Supplementary Table 1. Red and green bars (mean ± s.d.) correspond to the predicted ~~'Off'~~Off and ~~'On'~~On states, respectively. **a**~~-~~**c**, Regulatory program 'ZsYellow = NOT(rtTA) AND LacI-Krab'. **a**, From left to right~~, top to bottom **Kobi, please make the bottom as panel b and revise accordingly. The caption is currently confusing**~~: circuit schematics; representative microscopy snapshots and quantitative performance of the circuit with CMV-driven output~~; surface~~. **b**, Surface plot of control-corrected ZsYellow output as a function of ~~microRNA~~miR-FF3 and FF4 levels judged ~~from~~ by the levels of DsRed and AmCyan, respectively~~; images~~. **c**, Images and quantitative analysis of the circuit with EF1a-driven output. ~~**b**~~**d**, Regulatory program 'ZsYellow = NOT(rtTA) AND NOT (Rheo)' implemented with EF1a-driven output. Left: circuit schematics; right: anticipated circuit behavior, microscopy images and quantitative analysis.

Figure 3 | Experimental implementation of a three-input regulatory program. Plasmid amounts are given in Supplementary Table 1. From left to right: circuit schematics; table of TF input states, the expected outputs and fluorescent output levels of ZsYellow; microscopy images of ZsYellow output; and quantitative output intensity as obtained by FACS analysis. Red and green bars (mean ± s.d.) indicate anticipated ~~'Off'~~Off and ~~'On'~~On states, respectively.