LETTERS

A genome-wide *Drosophila* RNAi screen identifies DYRK-family kinases as regulators of NFAT

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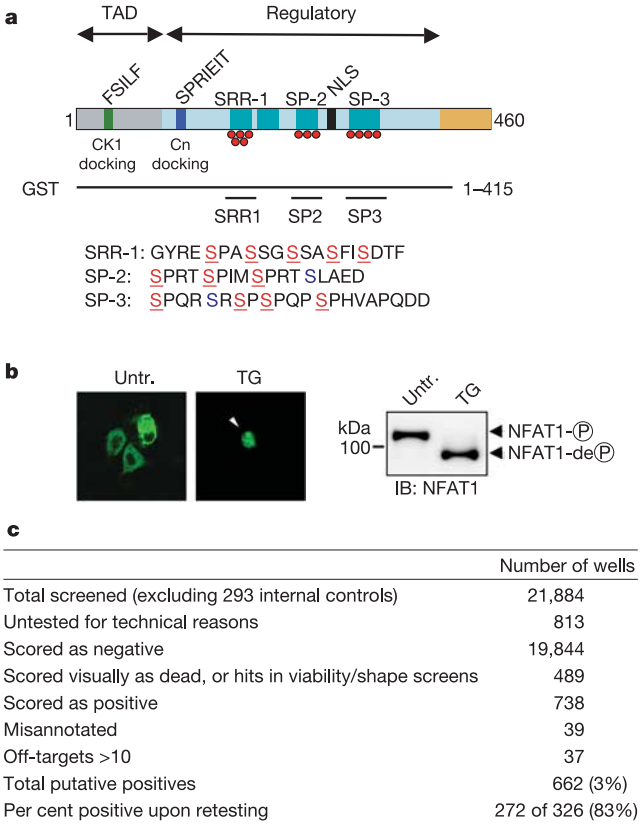
Precise regulation of the NFAT (nuclear factor of activated T cells) family of transcription factors (NFAT1–4) is essential for vertebrate development and function[1]. In resting cells, NFAT proteins are heavily phosphorylated and reside in the cytoplasm; in cells exposed to stimuli that raise intracellular free Ca^{2+} levels, they are dephosphorylated by the calmodulin-dependent phosphatase calcineurin and translocate to the nucleus[1]. NFAT dephosphorylation by calcineurin is countered by distinct NFAT kinases, among them casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3)[1–5]. Here we have used a genome-wide RNA interference (RNAi) screen in *Drosophila*[6,7] to identify additional regulators of the signalling pathway leading from Ca^{2+}–calcineurin to NFAT. This screen was successful because the pathways regulating NFAT subcellular localization (Ca^{2+} influx, Ca^{2+}–calmodulin–calcineurin signalling and NFAT kinases) are conserved across species[8,9], even though Ca^{2+}-regulated NFAT proteins are not themselves represented in invertebrates. Using the screen, we have identified DYRKs (dual-specificity tyrosine-phosphorylation regulated kinases) as novel regulators of NFAT. DYRK1A and DYRK2 counter calcineurin-mediated dephosphorylation of NFAT1 by directly phosphorylating the conserved serine-proline repeat 3 (SP-3) motif of the NFAT regulatory domain, thus priming further phosphorylation of the SP-2 and serine-rich region 1 (SRR-1) motifs by GSK3 and CK1, respectively. Thus, genetic screening in *Drosophila* can be successfully applied to cross evolutionary boundaries and identify new regulators of a transcription factor that is expressed only in vertebrates.

To validate the use of genome-wide RNAi screening in *Drosophila* to identify regulators of the Ca^{2+}–calcineurin–NFAT signalling pathway, we used an NFAT–GFP (green fluorescent protein) fusion protein[10] containing the entire regulatory domain of NFAT1 (Fig. 1a). This domain bears >14 phosphorylated serines, 13 of which are dephosphorylated by calcineurin[4]. Five of the thirteen serines are located in the SRR-1 motif, which controls exposure of the nuclear localization sequence (NLS) and is a target for phosphorylation by CK1 (refs 4, 5); three are located in the SP-2 motif, which can be phosphorylated by GSK3 after a priming phosphorylation by protein kinase A (PKA)[2,5]; and four are located in the SP-3 motif, for which a relevant kinase had yet to be identified at the time this study was initiated. The SP-2 and SP-3 motifs do not directly regulate the subcellular localization of NFAT1, but their dephosphorylation increases both the probability of NLS exposure and the affinity of NFAT for DNA[1,4,5,11]. NFAT–GFP was correctly regulated in *Drosophila* $S2R^+$ cells (Fig. 1b; see also Supplementary Fig. 1): it was phosphorylated and localized to the cytoplasm under resting conditions; it became dephosphorylated and translocated to the nucleus with appropriate kinetics in response to Ca^{2+} store depletion with the sarcoplasmic/endoplasmic reticulum ATPase (SERCA) inhibitor thapsigargin; and its dephosphorylation and nuclear translocation were both sensitive to the calcineurin inhibitor cyclosporin



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	Number of wells
Total screened (excluding 293 internal controls)	21,884
Untested for technical reasons	813
Scored as negative	19,844
Scored visually as dead, or hits in viability/shape screens	489
Scored as positive	738
Misannotated	39
Off-targets >10	37
Total putative positives	662 (3%)
Per cent positive upon retesting	272 of 326 (83%)

Figure 1 | The NFAT regulatory domain and the genome-wide RNAi screen in *Drosophila*. **a**, Diagram of the NFAT1 regulatory domain. Red circles or red and underlined residues show phosphorylated serine residues in the SRR-1, SP-2 and SP-3 motifs. NLS, nuclear localization signal; TAD, transactivation domain. **b**, NFAT is regulated by Ca^{2+} entry in *Drosophila* cells. $S2R^+$ cells expressing GFP–NFAT1 were left untreated (Untr.) or treated with thapsigargin (TG, 1 μM, 30 min) and analysed by microscopy (left). Lysates were analysed by immunoblotting (IB) with anti-NFAT1 (right). P, phosphorylated; deP, dephosphorylated. **c**, Tabulation of the results of the primary screen.

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A (CsA). S2R$^+$ cells treated with limiting amounts of thapsigargin displayed intermediate phosphorylated forms of NFAT–GFP, most likely reflecting progressive dephosphorylation of serines within individual conserved motifs of the regulatory domain[4,5] (Supplementary Fig. 1b). Depletion of the primary NFAT regulator, calcineurin, by RNAi in S2R$^+$ cells inhibited thapsigargin-dependent dephosphorylation and nuclear import of NFAT–GFP (Supplementary Fig. 1c–e and Supplementary Table 2). Thus, the major pathways regulating NFAT phosphorylation and subcellular localization—store-operated Ca^{2+} influx, calcineurin activation and NFAT phosphorylation/dephosphorylation—are conserved in *Drosophila* and appropriately regulate vertebrate NFAT.

We performed a genome-wide RNAi screen[7] on unstimulated S2R$^+$ cells, and scored visually for aberrant nuclear localization of NFAT–GFP (Fig. 1c; see Methods and Supplementary Information). Positive candidates obtained in the screen (Supplementary Table 1) include Na^+/Ca^{2+} exchangers and SERCA Ca^{2+} ATPases, the knockdown of which would be expected to increase basal levels of intracellular free Ca^{2+} ($[Ca^{2+}]_i$)[12]; the scaffold protein Homer, which has been linked to Ca^{2+} influx and Ca^{2+} homeostasis[13]; stromal interaction molecule (STIM), a recently identified regulator of store-operated Ca^{2+} influx[14–16] (Supplementary Fig. 2); and several protein kinases that control NFAT function either directly via phosphorylation or indirectly via basal $[Ca^{2+}]_i$ levels, calcineurin activity, or other kinases (Supplementary Tables 1 and 3). To identify kinases that directly phosphorylate the NFAT regulatory domain, we expressed Flag-tagged human homologues of selected *Drosophila* kinases in HEK293 cells, and tested anti-Flag immunoprecipitates in an *in vitro* kinase assay for their ability to phosphorylate a GST–NFAT1(1–415) fusion protein. Three kinases—protein kinase cGMP-dependent (PRKG1), DYRK2 and interleukin (IL)-1 receptor-associated kinase 4 (IRAK4)—showed strong activity in this assay (Fig. 2a, top; CK1-α and CK1-ε are positive controls). In cells, only DYRK2 countered the dephosphorylation of NFAT–GFP by calcineurin (Fig. 2b), even though both PRKG1 and DYRK2 were expressed at high levels (Fig. 2a, bottom). CD4$^+$ T_H1 cells isolated from *Irak4*$^{-/-}$ mice[17] showed normal NFAT1 dephosphorylation, rephosphorylation and nuclear transport compared to control T_H1 cells (Supplementary Fig. 3a, b). We therefore focused on DYRK-family kinases as potential direct regulators of NFAT.

DYRKs constitute an evolutionarily conserved family of proline- or arginine-directed protein kinases belonging to the CMGC family of cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), GSK and CDK-like kinases (CLKs)[18,19]. The DYRK family has multiple members (Supplementary Fig. 4a) that can be predominantly nuclear (DYRK1A and DYRK1B) or cytoplasmic (DYRK2–4 and homeodomain interacting protein kinase 3 (HIPK3)/DYRK6)[20]. RT–PCR and western blotting suggested that DYRK1A and DYRK2 were major representatives of nuclear and cytoplasmic DYRKs, respectively, in Jurkat T cells (Supplementary Fig. 4b)—a conclusion supported by several additional observations. First, overexpression of DYRK2 prevented dephosphorylation of NFAT–GFP after ionomycin treatment (Fig. 2b, lanes 5–8); overexpression of wild-type DYRK2, but not a kinase-dead mutant of DYRK2 (refs 21, 22), prevented NFAT nuclear localization in thapsigargin-treated cells (Fig. 2c). The slower-migrating form of NFAT in lanes 7 and 8 of Fig. 2b might indicate that DYRK2, a proline-directed kinase[18], acted in part by phosphorylating the SPRIEITP docking sequence on NFAT1 (refs 1, 10) and thereby blocking the calcineurin–NFAT interaction. However, DYRK was still effective when the potential DYRK phosphorylation sites in the docking sequence were eliminated by substituting HPVIVITGP for SPRIEITPS[10] (Supplementary Fig. 5a). Second, both wild-type and kinase-dead DYRK co-immunoprecipitated with NFAT1 (Supplementary Fig. 5b). Third, depletion of the DYRK-family candidate CG40478 in S2R$^+$ cells did not affect—and DYRK2 overexpression in Jurkat T cells only slightly diminished—Ca^{2+} mobilization in response to thapsigargin (Supplementary Fig. 6a, b). Finally, and most importantly, depletion of endogenous DYRK1A with DYRK1A-specific short interfering RNAs (siRNA) in HeLa cells stably expressing NFAT–GFP increased the rate and extent of NFAT1 dephosphorylation and nuclear import while slowing rephosphorylation and nuclear export (Fig. 3a, b). These results show that DYRK1A and DYRK2 are physiological negative regulators of NFAT activation in cells. The absence of basal NFAT dephosphorylation in DYRK1A-depleted cells may reflect both the expression of other DYRK family members in human cells (Supplementary Fig. 4b) and the predominantly nuclear localization of DYRK1A (refs 19, 20).

DYRKs are direct NFAT1 kinases that selectively phosphorylate the SP-3 motif, but nevertheless control the overall phosphorylation

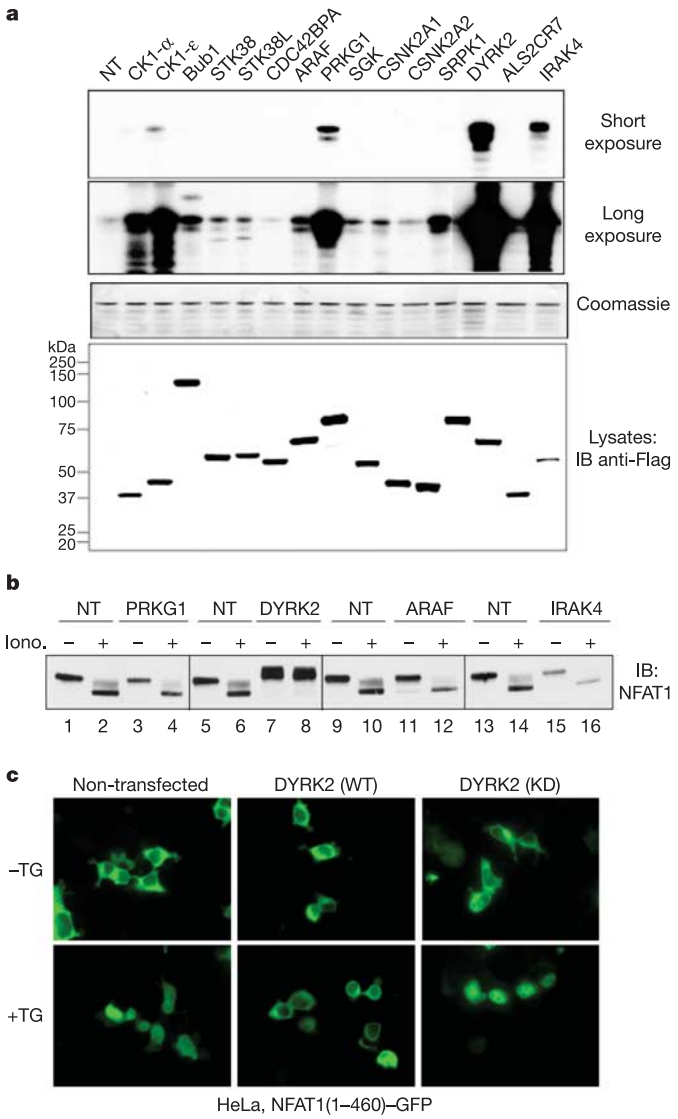

Figure 2 | Screening for NFAT phosphorylation: DYRK kinase negatively regulates NFAT activation. a, Phosphorylation of the NFAT regulatory domain. Flag immunoprecipitates of human homologues of selected *Drosophila* kinases were tested for GST–NFAT1(1–415) phosphorylation, as assessed by autoradiography (top and middle panels). Kinase expression was verified by immunoblotting using anti-Flag antibody (bottom panel). NT, not transfected. **b**, DYRK2 overexpression blocks NFAT–GFP dephosphorylation in ionomycin-treated cells, assessed by immunoblotting of cell lysates with anti-NFAT1. **c**, Overexpression of wild-type (WT), but not kinase-dead (KD), DYRK2 blocks NFAT–GFP nuclear translocation. NFAT–GFP localization (green) was examined by fluorescence microscopy in untreated (−TG) and thapsigargin-treated (+TG) cells.

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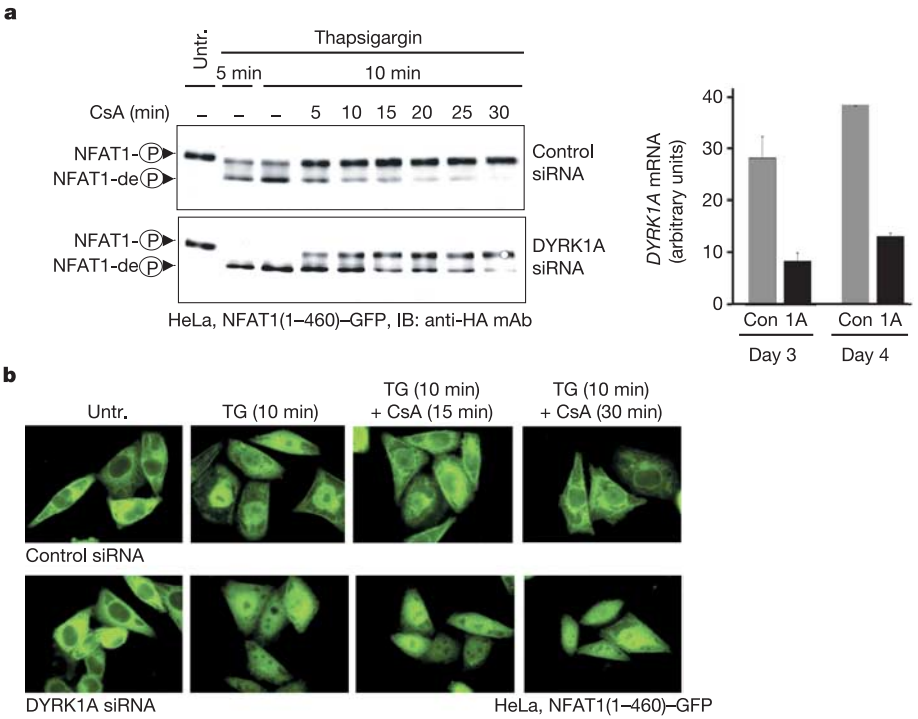

Figure 3 | Depletion of endogenous DYRK1A potentiates NFAT activation. **a**, Depletion of DYRK1A delays re-phosphorylation of nuclear NFAT. HeLa cells stably expressing NFAT–GFP were transfected with control (Con) or DYRK1A-specific (1A) siRNAs, and treated with 1 μM thapsigargin (TG) followed by 20 nM CsA. Phosphorylation status of NFAT–GFP was assessed by western blotting with anti-HA (left). *DYRK1A* messenger RNA levels were assessed 3 and 4 days after transfection by real-time PCR (right). Results show average ±s.d. of three independent experiments. **b**, Depletion of DYRK1A delays NFAT nuclear export. NFAT–GFP localization (green) was examined by fluorescence microscopy in siRNA-transfected cells.

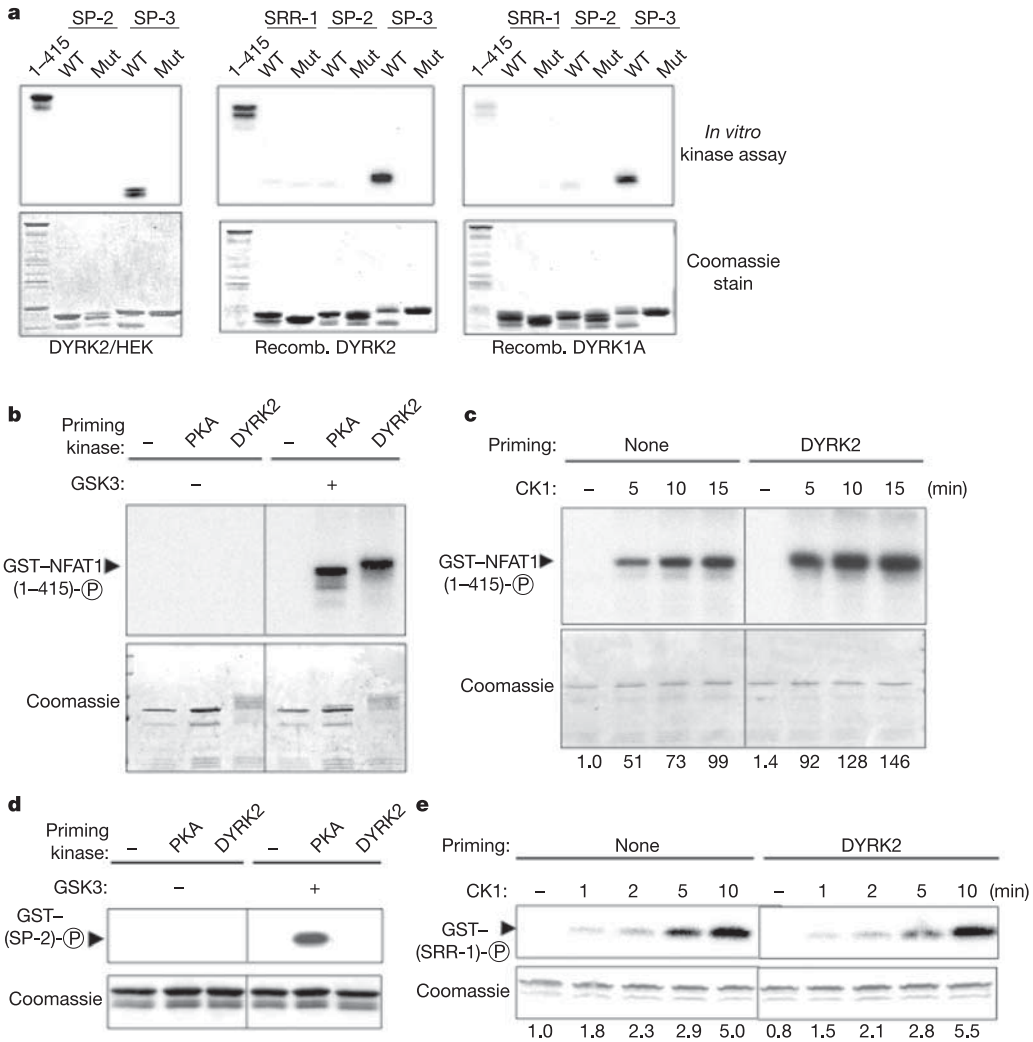

Figure 4 | Pre-phosphorylation by DYRK primes the NFAT regulatory domain for subsequent phosphorylation by GSK3 and CK1. **a**, Immunopurified DYRK2, recombinant DYRK2 or recombinant DYRK1A was incubated with GST–NFAT1(1–415); with the indicated GST–peptide fusion GST–SRR-1, GST–SP-2 or GST–SP-3 (WT); or with a corresponding GST–peptide fusion (Mut) carrying serine-to-alanine replacements at the residues known to be phosphorylated in cells (Fig. 1a). **b**, DYRK2 and PKA prime for GSK3 phosphorylation of the NFAT1 regulatory domain. **c**, DYRK2 potentiates CK1 phosphorylation of the NFAT1 regulatory domain (quantification below). **d**, DYRK2 does not prime for GSK3 phosphorylation of the SP-2 motif. **e**, DYRK2 does not prime for CK1 phosphorylation of the SRR-1 motif (quantification below).

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of NFAT1 (Fig. 4). Flag-tagged DYRK2 expressed in HEK cells, as well as bacterially expressed recombinant DYRK1A and DYRK2, phosphorylated peptides corresponding to the SP-3 motif of the NFAT1 regulatory domain *in vitro*, but did not phosphorylate SRR-1 or SP-2 peptides or an SP-3 peptide with serine to alanine substitutions in the known phosphoserine residues[4] (Fig. 4a). Two serine residues (underlined) in the SP-3 motif (SPQR SRSPSPQPSPHVAPQDD) fit the known sequence preference of DYRK kinases (R(x)xx(S/T)(P/V))[23–25], and both are known to be phosphorylated in cells[4] (Fig. 1a). DYRK is reported to prime for GSK3-mediated phosphorylation of eukaryotic initiation factor 2B-ε (eIF2B-ε) and the microtubule-associated protein tau[25], as well as for GSK3- and CK1-mediated phosphorylation of OMA-1 (refs 26 and 27). We therefore investigated whether DYRK kinases could also prime for GSK3- and CK1-mediated phosphorylation of NFAT1. Pre-phosphorylation of the NFAT1 regulatory domain by DYRK2 led to robust phosphorylation by GSK3 and induced the mobility shift characteristic of phosphorylation of the SP-2 and SP-3 motifs[4]; this shift was not observed after pre-phosphorylation by PKA (Fig. 4b). Furthermore, pre-phosphorylation by DYRK2 accelerated CK1-mediated phosphorylation of GST–NFAT1(1–415) by at least two-fold (Fig. 4c). In contrast, DYRK2 did not prime phosphorylation of the SP-2 peptide by GSK3 (Fig. 4d) nor the SRR-1 peptide by CK1 (Fig. 4e), consistent with the fact that neither motif is a substrate for DYRK (Fig. 4a). This 'discontiguous' priming mechanism is distinct from conventional priming, which requires phosphorylation at +4 and −3 for GSK3 and CK1, respectively[28,29]. A less likely interpretation is that the conventional priming sites for CK1 and GSK3 are efficiently phosphorylated by DYRK in the context of the GST–NFAT1(1–415) protein, although they are not phosphorylated in the peptide context.

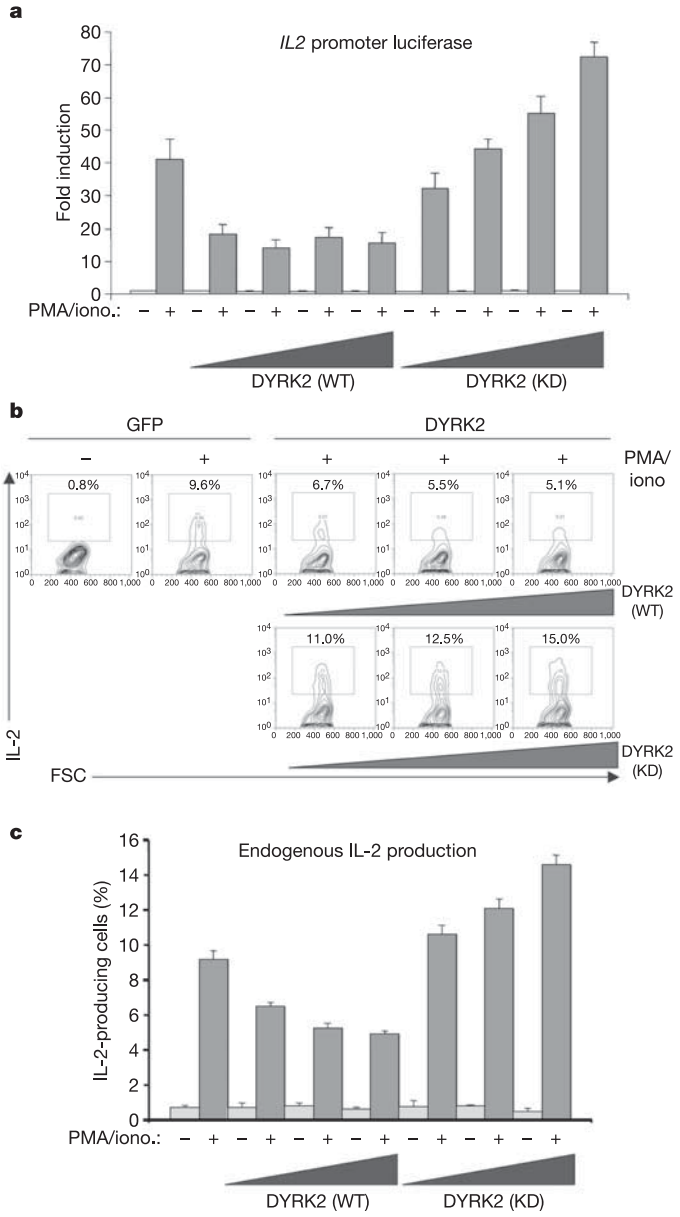

Figure 5 | DYRK2 inhibits IL-2 transcription. a, DYRK2 inhibits *IL2* promoter activity. Jurkat T cells, co-transfected with *Renilla* and *IL2* promoter-driven luciferase reporters and wild-type or kinase-dead DYRK2 expression plasmids (5, 10, 15, 20 μg), were left untreated or stimulated with PMA/ionomycin (iono). Results show average ±s.d. of three independent experiments. **b**, DYRK2 inhibits endogenous IL-2 expression. IL-2 expression was evaluated by intracellular cytokine staining and flow cytometry in GFP^+ Jurkat T cells transfected with GFP and wild-type or kinase-dead DYRK2 expression plasmids (10, 20, 30 μg). **c**, Quantification of **b** (average ±s.d. of three independent experiments).

We used the kinase-dead mutant of DYRK2 to show that DYRK regulates the transcriptional activity of NFAT. Wild-type DYRK2 strongly diminished NFAT-dependent activity, whereas the kinase-dead mutant increased NFAT-dependent luciferase activity of the *IL2* promoter (Fig. 5a); an NFAT-activating protein 1 (AP-1) reporter (data not shown); or an AP-1-independent promoter, the κ3 site of the tumour-necrosis factor-α (TNF-α) promoter (Supplementary Fig. 6c). Similarly, wild-type DYRK2 diminished production of endogenous IL-2 by stimulated Jurkat T cells, whereas kinase-dead DYRK2 had the opposite effect (Fig. 5b, c).

Our data indicate that DYRK is a key kinase that regulates NFAT1 phosphorylation. DYRK, GSK3 and CK1 target completely distinct motifs of the NFAT1 regulatory domain, but DYRK-mediated phosphorylation of the SP-3 motif primes for further phosphorylation of the distinct SRR-1 and SP-2 motifs by CK1 and GSK3, respectively, thus facilitating complete phosphorylation and deactivation of NFAT1. This mechanism, which we term 'discontiguous priming', is reminiscent of that recently proposed for *Caenorhabditis elegans* oocyte maturation protein 1 (OMA-1), in which phosphorylation of Thr 239 by the DYRK-family kinase minibrain kinase 2 (MBK-2) potentiates GSK3-mediated phosphorylation of Thr 339 (refs 26, 27). It is likely that DYRK2, DYRK3 and DYRK4, which are localized to the cytoplasm[19], function primarily as 'maintenance' kinases that sustain the phosphorylation status of cytoplasmic NFAT in resting cells, whereas DYRK1A and DYRK1B, which are localized to the nucleus[19,20], re-phosphorylate nuclear NFAT and promote its nuclear export. Notably, NFAT dephosphorylation may also proceed through a sequential mechanism, with dephosphorylation of the SRR-1 motif promoting dephosphorylation of the SP-2 and SP-3 motifs by increasing their accessibility to calcineurin[4]. *DYRK1A* and the endogenous calcineurin regulator *DSCR1*/*RCN*/calcipressin-1 are both localized to the Down's syndrome critical region on human chromosome 21; thus, overexpression of these negative regulators of NFAT could contribute—by inhibiting NFAT activation—to the neurological and immunological developmental anomalies observed in individuals with chromosome 21 trisomy[30].

We have shown that genome-wide RNAi screening in *Drosophila* is a valid and powerful strategy for exploring novel aspects of signal transduction in mammalian cells, provided that key members of the signalling pathway are evolutionarily conserved and represented in the *Drosophila* genome. We have used the method to identify conserved regulators of the purely vertebrate transcription factor NFAT; to our knowledge, this is the first example of a genome-wide RNAi screen that crosses evolutionary boundaries in this manner. It is likely that conserved aspects of the regulation of other mammalian processes will also be successfully defined by developing assays in *Drosophila* cells.

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METHODS

Detailed methods are presented in Supplementary Information.

The genome-wide primary screen. Methods were adapted from refs 6 and 7. A total of 10^4 S2R$^+$ cells were added to each well containing 0.25 μg double-stranded (ds)RNAs in 10 μl serum-free medium and incubated for 1 h at 26 °C. The cells were then transiently transfected with NFAT1(1–460)–GFP expression plasmid[10] (10 ng) in Schneider's medium (Invitrogen) (30 μl). After incubation for 48–72 h at 26 °C, the cells were fixed and stained with 4,6-diamidino-2-phenylindole (DAPI), and the coincident GFP and DAPI images were acquired by an automated camera from three different locations in each well. A total of 58 384-well plates were analysed, containing a total of 21,884 wells into which individual dsRNAs had been arrayed. The confirmatory screening on the 699 potentially positive candidates from the primary screen was performed essentially as described for the primary screen, except that S2R$^+$ cells stably transfected with NFAT1(1–460)–GFP were used.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions The *Drosophila* RNAi screen was designed, validated and performed by Y.G., and positive candidates were identified through visual screening performed by Y.G., J.N. and H.O. S. Sharma identified DYRK as a functional NFAT kinase through biochemical and functional analyses of the human homologues of candidate *Drosophila* kinases, drawing in part on unpublished data and reagents provided by H.O. Selected other candidates were investigated in detail by Y.G., S. Sharma, D.B. and S. Srikanth. A.I. and S.F. performed measurements of intracellular calcium concentration in mammalian cells. J.N. and B.T. were responsible for bioinformatic analysis. P.G.H. and A.R. provided overall direction and supervised project planning and execution.

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