aware of this. Moreover, there is a level of subjectivity in their conclusions, as the nature of each individual piece of data in-and-of-itself, can be confounding. For instance, the breakdown of Hox clustering has been observed in several bilaterian groups [4,5,10] and in the tunicate *Oikopleura* the Hox cluster has diverged so dramatically that none of its Hox genes are linked [3]; yet, all of these animals show high axial complexity and a similar Hox gene expression pattern along the body axis. Moreover, the difficulty in assigning strict Hox classes to the cnidarian Hox genes could be a result of the very old age of this lineage as well as the vagaries of using the short homeodomains (60 amino acids) for phylogenetic inference [11]. Differences in the Hox repertoire between *Nematostella* and *Eleutheria* could be significant, but a similar situation has been seen in tunicates, between *Ciona* and *Oikopleura* [3]. And with respect to the expression patterns, while we should be able to recognize when colinear patterns exist, the very deep divergence time of the cnidarian lineage may have wiped out their vestiges. Differences in expression pattern seen amongst the different taxa for the same Hox gene have also been observed in bilaterians such as fishes [12]. Finally, might the cnidarian Hox repertoire have been used in a non-colinear fashion in the myriad morphological differences seen in the group, equivalent to the stunning innovations involving Hox gene expression that were observed in a cephalopod [13]? The conclusions by Kamm *et al.* [8] are of course strongest when the data are considered as a whole, and are certainly thought-provoking. It will be of extreme interest to see how well their conclusions hold up as genomes of other cnidarians and primitive metazoans such as sponges, placozoans and acoel flatworms are examined and sequenced.

References

1. McGinnis, W., and Krumlauf, R. (1992). Homeobox genes and axial patterning. Cell *68*, 283–302.
2. Ronshaugen, M., McGinnis, N., and McGinnis, W. (2002). Hox protein mutation and macroevolution of the insect body plan. Nature *415*, 914–917.
3. Edvardsen, R.B., Seo, H.C., Jensen, M.F., Mialon, A., Mikhaleva, J., Bjordal, M., Cartry, J., Reinhardt, R., Weissenbach, J., Wincker, P., *et al.* (2005). Remodelling of the homeobox gene complement in the tunicate Oikopleura dioica. Curr. Biol. *15*, R12–R13.
4. Ikuta, T., Yoshida, N., Satoh, N., and Saiga, H. (2004). Ciona intestinalis Hox gene cluster: Its dispersed structure and residual colinear expression in development. Proc. Natl. Acad. Sci. USA *101*, 15118–15123.
5. Negre, B., Casillas, S., Suzanne, M., Sanchez-Herrero, E., Akam, M., Nefedov, M., Barbadilla, A., de Jong, P., and Ruiz, A. (2005). Conservation of regulatory sequences and gene expression patterns in the disintegrating Drosophila Hox gene complex. Genome Res. *15*, 692–700.
6. Cameron, R.A., Rowen, L., Nesbitt, R., Bloom, S., Rast, J.P., Berney, K., Arenas-Mena, C., Martinez, P., Lucas, S., Richardson, P.M., *et al.* (2006). Unusual gene order and organization of the sea urchin hox cluster. J. Exp. Zoolog. B Mol. Dev. Evol. *306*, 45–58.
7. Powers, T.P., and Amemiya, C.T. (2004). Evidence for a Hox14 paralog group in vertebrates. Curr. Biol. *14*, R183–R184.
8. Kamm, K., Schierwater, B., Jakob, W., Dellaporta, S.L., and Miller, D.J. (2006). Axial patterning and diversification in the cnidaria predate the hox system. Curr. Biol. *16*, 920–926.
9. Finnerty, J.R., Pang, K., Burton, P., Paulson, D., and Martindale, M.Q. (2004). Origins of bilateral symmetry: Hox and dpp expression in a sea anemone. Science *304*, 1335–1337.
10. Aboobaker, A.A., and Blaxter, M.L. (2003). Hox gene loss during dynamic evolution of the nematode cluster. Curr Biol. *13*, 37–40.
11. Ferrier, D.E., Minguillon, C., Holland, P.W., and Garcia-Fernandez, J. (2000). The amphioxus Hox cluster: deuterostome posterior flexibility and Hox14. Evol. Dev. *2*, 284–293.
12. Amores, A., Suzuki, T., Yan, Y.L., Pomeroy, J., Singer, A., Amemiya, C., and Postlethwait, J.H. (2004). Developmental roles of pufferfish Hox clusters and genome evolution in ray-fin fish. Genome Res. *14*, 1–10.
13. Lee, P.N., Callaerts, P., De Couet, H.G., and Martindale, M.Q. (2003). Cephalopod Hox genes and the origin of morphological novelties. Nature *424*, 1061–1065.

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Calcium Influx: Beyond 'Current' Biology

A novel, cell-surface protein essential for Ca^{2+} release-activated Ca^{2+} (CRAC) channel function has been identified through independent genome-wide screens. This huge advance will enable molecular dissection of the CRAC channel complex, moving the field beyond I_{CRAC} signature to structure.

Lisbeth C. Robinson and Jonathan S. Marchant

In the years since the phenomenon of intracellular Ca^{2+} store-operated Ca^{2+} entry was first proposed, two black boxes have frustrated researchers: the molecular identity of the store-operated channels (SOCs) that mediate Ca^{2+} entry in response to Ca^{2+} store depletion, and the mechanism by which Ca^{2+} store depletion is communicated to these elusive SOCs at the cell surface (reviewed in [1–5]). Within the last year, however, immense progress has been made towards solving both these problems. This time last year, STIM1 (stromal interaction molecule 1) was identified as a prime candidate for the Ca^{2+} sensor that couples Ca^{2+} store depletion to SOC activation [6,7]. Now, two research groups [8,9] have independently converged upon a novel transmembrane protein essential for the function of the best defined SOC — the Ca^{2+}-release activated Ca^{2+} (CRAC) channel that has been electrophysiologically well-characterized in T cells and several other cell types [3,4]. Whether this new protein proves to be the entire CRAC channel itself, an essential subunit or an obligate cell-surface regulator, the long search for a molecular identity underpinning I_{CRAC} is over.

The major catalyst for both of these developments has been the application of genome-wide RNA

interference (RNAi) screening, performed mostly in *Drosophila* cell lines [6,8,9] using available libraries that capitalize on the robustness of RNAi effected by long dsRNAs and the lower redundancy of the *Drosophila* genome relative to mammalian models. In a doublet of *Nature* papers from Anjana Rao's group [8,10], this RNAi screening approach was executed in parallel with another screen — a more classical linkage analysis — in a pedigree harbouring two siblings with a rare, hereditary severe combined immunodeficiency (SCID), which in these specific patients has been attributed to an absence of I_{CRAC} [11–13]. Single nucleotide polymorphism (SNP) mapping analysis of SCID family members first delimited six genomic regions, subsequently refined to a single, multigenic stretch containing a human homologue of a candidate also identified in the *Drosophila* RNAi screen. This synergistic convergence of two unbiased, genome-wide screens — in humans and flies — to identify a novel gene that, first, is naturally mutated in the SCID pedigree, and, second, complements the SOC influx and I_{CRAC} deficit in SCID T-cell lines, represents a truly comprehensive body of work spanning bench-to-bedside.

Gwack *et al.* [10] detail the results of the RNAi screens, in which defects in resting localization of a GFP-tagged NFAT1 construct, as well as nuclear shuttling of the same construct in thapsigargin-stimulated cells were scored. This was an audacious approach given the absence of the Ca^{2+}-dependent NFAT family members (NFAT1–4) in invertebrates [14]. However, functional conservation of other key components of this signalling pathway in *Drosophila* (an I_{CRAC}-like current [15], calcineurin and other NFAT maintenance/export kinases [10]) ensured that the sustained Ca^{2+} influx signal evoked by Ca^{2+} store depletion triggered dephosphorylation and nuclear translocation of the vertebrate NFAT reporter [8], recapitulating the membrane-to-nucleus signaling pathway impaired in the SCID T cells. A

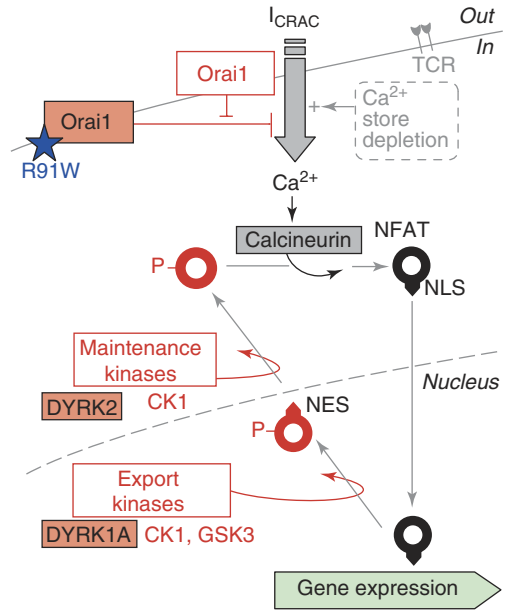

Figure 1. Role of Orai1 in T-cell function.

Schematic summary of the results of [8–10], which identify Orai1 as an essential component or regulator of I_{CRAC}. Top, activation of the T-cell receptor (TCR) causes depletion of intracellular Ca^{2+} stores and activation of the calcium-release activated Ca^{2+} (CRAC) channel current known as I_{CRAC}. In a T-cell line lacking I_{CRAC}, isolated from patients with a hereditary severe combined immunodeficiency (SCID), Feske *et al.* [8] identify Orai1 as an integral component of the CRAC channel complex and show that I_{CRAC} is abrogated by a missense (R91W) mutation in the afflicted patients. Expression of wild-type Orai1 complements the CRAC channel deficit, restoring store-operated Ca^{2+} influx and I_{CRAC} in the SCID T cells. Bottom, downstream of I_{CRAC}. Ca^{2+}-bound calmodulin activates calcineurin to dephosphorylate NFAT (black, dephosphorylated form) and expose a nuclear localization sequence (NLS). Sustained Ca^{2+} influx mediated by I_{CRAC} is necessary to effect a prolonged NFAT translocation that regulates genes crucial for T-cell activation [12]. NFAT shuttling is also controlled by a variety of 'maintenance' kinases (e.g. casein kinase 1, CK1), which phosphorylate NFAT at multiple sites (red, phosphorylated form) to keep it in the cytoplasm, and 'export' kinases (e.g. CK1 and glycogen synthase kinase 3, GSK3), which rephosphorylate nuclear NFAT to terminate gene transcription and expose a nuclear export signal (NES). Gwack *et al.* [10] identify the DYRK-family kinases as new regulators of NFAT shuttling (see also [16]), functioning as both export (DYRK1A) and maintenance (DYRK2) kinases that phosphorylate the NFAT serine-rich SP-3 motif.

variety of candidates (~1 in 40 tested dsRNAs), spanning the expected and unexpected, emerged from these assays and Rao's group have initially focused on results stemming from two *Drosophila* gene 'hits': *olf186-F*, the gene essential for store-operated Ca^{2+} entry [8] and *CG40478*, a member of the DYRK family of dual-specificity protein kinases, now revealed as novel regulators of NFAT shuttling ([10], see also [16]). While this commentary focuses on the former discovery, it would be remiss not to highlight recent data linking developmental facets of Down's syndrome to altered NFAT nuclear residency mediated by increased levels of DYRK1A [16]. Clearly, this proved a very informative RNAi screen.

The *Drosophila* gene *olf186-F* has three human homologues, one of which (*FLJ14466,* see nomenclature [17]) localized within the genomic region delimited by the SCID linkage analysis. DNA sequencing revealed that two identical missense mutations in *FLJ14466* were harboured in this specific SCID pedigree, and two copies of the mutated allele were inherited by the afflicted brothers [8]. Feske *et al.* [8] christened the members of this novel gene family *ORAI1*, *ORAI2* and *ORAI3* after the Homeric Hours (Ωραι, [18]), guardians of the gates of heaven, who regulated passage between heaven and Olympus, and the earth below. *ORAI1* (*FLJ14466*) encodes a ~300 amino-acid protein (Orai1) containing four predicted transmembrane domains separating cytoplasmic amino and catboxyl termini. The SCID mutation (resulting in an arginine to tryptophan substitution, R91W) lies at the start of the first predicted transmembrane domain, impairing I_{CRAC} function, but not targeting of Orai1 to the cell surface [8]. Expression of wild-type Orai1 (but not Orai1[R91W]) was sufficient to complement the store-operated Ca^{2+} influx and I_{CRAC} deficit in SCID T cells, uniquely and fully restoring

a Ca^{2+} current with the electrophysiological and pharmacological signature of I_{CRAC} [8,13]. Independently, Vig *et al.* [9] also identified *olf186-F* from a high throughput Ca^{2+} imaging-based RNAi screen, again in *Drosophila* S2R+ cells. Their data underscore the conclusions of the study described above, but additionally demonstrate the effects of manipulating Orai1 levels (named CRACM1 in [9]) in various cell lines. Their electrophysiological measurements show that siRNA-mediated silencing of endogenous Orai1 abolishes I_{CRAC}, but Orai1 overexpression does not potentiate I_{CRAC} amplitude.

It is not surprising that mutational ablation of I_{CRAC} in T cells results in impaired immunocompetency. CRAC channels probably constitute the sole route for Ca^{2+} entry in T cells following antigen stimulation of the T-cell receptor [19], and the resultant sustained Ca^{2+} entry signals are crucial for regulating gene expression integral to T-cell activation ([12], see Figure 1). However, it is worth commenting on the observations that, first, heterozygote carriers of the R91W mutation are disease-free (impairment in Ca^{2+}-store dependent Ca^{2+} influx was demonstrable experimentally only under hypocalcemic conditions, [8]) and, second, that the mutation precipitates a specific cellular/tissue pathology (despite the widespread distribution of *ORAI1* mRNA [8]). Ultimately, both observations underscore the importance of expression level ('dosage'), in addition to functional redundancy in Ca^{2+} entry mechanisms ('diversity') in disease progression. That functional heterozygosity does not manifest T-cell dysfunction implies reserve capacity for I_{CRAC} functionality in T-cell behaviour (normally >5000 active CRAC channels per cell [19]). Moreover, in other cell types in these patients, where I_{CRAC} density is even lower [9], yet presumably critical for specific cellular activities, other mechanisms — perhaps expression of Orai2/Orai3 — must complement the I_{CRAC} deficit associated with Orai1[R91W], since the clinical presentation of the SCID brothers is predominantly one of immunodeficiency. Whatever the basis for this redundancy, the fact that loss of Orai1 function proved embryonically non-lethal was crucial to the success of this entire approach.

Discovery of the role of this novel gene family is a major leap forward for the Ca^{2+} signalling community [8,9]. *ORAI1* is annotated in the genomes of a variety of organisms that have agonist-releasable intracellular Ca^{2+} stores, and its sequence is well conserved. With the identification of Orai1, molecular dissection of its role in the CRAC channel complex can begin. Does Orai1 singularly constitute the CRAC channel or does it act as an obligate subunit of a heteromeric assembly with other proteins? Perhaps Orai2 and/or Orai3, which share >70% amino acid equivalency with Orai1, fulfill this role. Or, to take the strictest analogy with the mythological Ωραι, does Orai1 act as a discrete, yet essential, regulator (gatekeeper) of CRAC channel (the gate itself)? What about the relationship between Orai1 and STIM [6,7]? Live cell imaging data using evanescent wave microscopy correlates juxtamembrane puncta of translocated STIM1 with focal sites of Ca^{2+} entry resulting from CRAC channel activity [20]. Consequently, extrapolation of these new data [8,9] would predict Orai1 to be resident and locally activated within these zones. As these exciting new data strengthen our molecular grasp onto I_{CRAC} [6–9], answers to all these questions cannot be far away.

References

1. Putney, J.W., Jr. (1986). A model for receptor-regulated calcium entry. Cell Calcium *7*, 1–12.
2. Venkatachalam, K., van Rossum, D.B., Patterson, R.L., Ma, H.-T., and Gill, D.L. (2002). The cellular and molecular basis of store-operated calcium entry. Nat. Cell Biol. *4*, E263–E272.
3. Parekh, A.B., and Putney, J.W.J. (2005). Store-operated calcium channels. Physiol. Rev. *85*, 757–810.
4. Prakriya, M., and Lewis, R.S. (2003). CRAC channels: activation, permeation, and the search for a molecular identity. Cell Calcium *33*, 311–321.
5. Berridge, M.J. (1995). Capacitative calcium entry. Biochem. J. *312*, 1–11.
6. Roos, J., DiGregorio, P.J., Yeromin, A.V., Ohlsen, K., Lioudyno, M., Zhang, S., Safrina, O., Kozak, J.A., Wagner, S.L., Cahalan, M.D., *et al.* (2005). STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. J. Cell Biol. *169*, 435–445.
7. Liou, J., Kim, L.M., Heo, W.D., Jones, J.T., Myers, J.W., Ferrell, J.E., Jr., and Meyer, T. (2005). STIM is a Ca^{2+} sensor essential for Ca^{2+} store depletion triggered Ca^{2+} influx. Curr. Biol. *15*, 1235–1241.
8. Feske, S., Gwack, Y., Prakriya, M., Srikanth, S., Puppel, S.-H., Tanasa, B., Hogan, P.G., Lewis, R.S., Daly, M., and Rao, A. (2006). A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature *441*, 179–185.
9. Vig, M., Peinelt, C., Beck, A., Koomoa, D.L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., *et al.* (2006). CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. Science *312*, 1220–1223.
10. Gwack, Y., Sharma, S., Nardone, J., Tanasa, B., Iuga, A., Srikanth, S., Okamura, H., Bolton, D., Feske, S., Hogan, P.G., *et al.* (2006). A genome-wide Drosophila RNAi screen identifies DYRK-family kinases as regulators of NFAT. Nature *441*, 646–650.
11. Feske, S., Muller, J.M., Graf, D., Kroczek, R.A., Drager, R., Niemeyer, C., Baauerle, P.A., Peter, H.H., and Schlesier, M. (1996). Severe combined immunodeficiency due to defective binding of the nuclear factor of activated T cells in T lymphocytes of two male siblings. Eur. J. Immunol. *26*, 2119–2126.
12. Feske, S., Giltnane, J., Dometsch, R., Staudt, L.M., and Rao, A. (2001). Gene regulation mediated by calcium signals in T lymphocytes. Nat. Immunol. *2*, 316–324.
13. Feske, S., Prakriya, M., Rao, A., and Lewis, R.S. (2005). A severe defect in CRAC Ca^{2+} channel activation and altered K^+ channel gating in T cells from immunodeficient patients. J. Exp. Med. *202*, 651–662.
14. Graef, I.A., Gastier, J.M., Francke, U., and Crabtree, G.R. (2001). Evolutionary relationships among Rel domains indicate functional diversification by recombination. Proc. Natl. Acad. Sci. USA *98*, 5740–5745.
15. Yeromin, A.V., Roos, J., Stauderman, K.A., and Cahalan, M.D. (2004). A store operated calcium channel in Drosophila S2 cells. J. Gen. Physiol. 167–182.
16. Arron, J.R., Winslow, M.M., Polleri, A., Chang, C.-P., Wu, H., Gao, X., Neilson, J.R., Chen, L., Heit, J.J., Kim, S.K., *et al.* (2006). NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. Nature *441*, 595–600.
17. http://www.gene.ucl.ac.uk/nomenclature/data/get_data.php?hgnc_id=HGNC:25896
18. The Iliad, Homer. Book 5, line 749. (Loeb Classical Library, Harvard University Press).
19. Prakriya, M., and Lewis, R.S. (2002). Separation and characterization of currents through store-operated CRAC channels and Mg^{2+}-inhibited cation (MIC) channels. J. Gen Physiol. *119*, 487–507.
20. Luik, R.M., Wu, M.M., and Lewis, R.S. (2006). Colocalization of STIM1 and active CRAC channels after calcium store depletion in Jurkat cells. Biophys. J. *90*, 1558P (Abstract).

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