NFAT proteins: emerging roles in cancer progression

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Abstract | The roles of nuclear factor of activated T cells (NFAT) transcription factors have been extensively studied in the immune system. However, ubiquitous expression of NFAT isoforms in mammalian tissues has recently been observed, and a role for these transcription factors in human cancer is emerging. Various NFAT isoforms are functional in tumour cells and multiple compartments in the tumour microenvironment, including fibroblasts, endothelial cells and infiltrating immune cells. How do NFAT isoforms regulate the complex interplay between these compartments during carcinoma progression? The answers lie with the multiple functions attributed to NFATs, including cell growth, survival, invasion and angiogenesis. In addition to elucidating the complex role of NFATs in cancer, we face the challenge of targeting this pathway therapeutically.

Store-operated channels
Calcium channels in the plasma membrane that allow the influx of extracellular calcium in response to the emptying of intracellular calcium stores such as the ER. Also known as CRAC channels.

Rel family transcription factors
Transcription factors with an N-terminal Rel-homology domain that is responsible for nuclear localization, dimerization and DNA binding.

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The nuclear factor of activated T cells (NFAT) signalling axis is a vertebrate-specific pathway important for various cellular functions. NFATs are best characterized as transcription factors that induce genes important in cellular processes ranging from the development and activation of lymphocytes to the differentiation of cardiac muscle cells[1,2]. In the canonical pathway, first elucidated in immune cells, NFATs are activated as a result of calcium flux from endoplasmic reticulum stores and from the extracellular environment through the activation of store-operated channels in the plasma membrane. In the basal state NFATs are hyperphosphorylated in the cytoplasm. Subsequent to cell stimulation and calcium release, NFATs are dephosphorylated by the phosphatase calcineurin and translocate to the nucleus, where they cooperate with other factors and co-activators to promote *de novo* gene transcription.

The foundation of the NFAT research field was based on the original discovery that NFATs are inducible nuclear factors bound to the interleukin-2 (*IL-2*) promoter during the activation of T cells[3]. The importance of NFAT signalling is also highlighted by the fact that immunosuppressants such as cyclosporin A (CsA) and FK506, which specifically inactivate the canonical NFAT pathway, are widely used in the clinic to prevent organ transplant rejection. Since their discovery two decades ago, it has become increasingly clear that NFAT transcription factors are expressed not only in immune cells but in all cells and tissues. In this context, several recent key findings have pointed to important roles for NFATs in modulating phenotypes associated with malignancy and tumour progression. NFAT isoforms are overexpressed in human solid tumours and haematological malignancies[4,5] and seem to have roles in cancer cell-autonomous functions such as invasive migration, differentiation and the survival of cells in the tumour and its microenvironment. NFATs also seem to have a key role in tumour angiogenesis[6]. It is thought that understanding the parts played by NFATs in tumour progression will help the development of effective therapeutics that target the NFAT pathway in cancer progression and metastasis.

Primary structure of the NFAT family

In humans the NFAT family comprises five distinct gene products: NFAT1 (also known as NFATc2 and NFATp); NFAT2 (also known as NFATc1 and NFATc); NFAT3 (also known as NFATc4); NFAT4 (also known as NFATc3 and NFATx) and NFAT5 (also known as TonEBP and OREBP) (TABLE 1). As the name implies, NFATs were originally identified and characterized in immune cells, although it is now known that all isoforms are ubiquitously expressed. In addition, each isoform has alternative splice variants that differ in the amino and carboxyl termini[7–9]. The calcium-regulated isoforms NFAT1–4 share two conserved domains (FIG. 1): the Rel-homology region (RHR), so called because of its structural similarity to the DNA binding domain of Rel family transcription factors (also known as the nuclear factor-κB (NF-κB) family)[10], and the more moderately conserved NFAT homology region (NHR). NFAT5 has a distinct domain structure and retains only

At a glance

- Nuclear factor of activated T cells (NFAT) is a family of closely related transcription factors that are ubiquitously expressed in mammalian cells and tissues. NFAT1–4 are regulated by the calcium-sensitive phosphatase calcineurin, which induces nuclear translocation and transcriptional activation.
- The transcriptional activity of NFATs is primarily regulated by phosphorylation that in turn determines subcellular localization. Maintenance kinases such as dual specificity tyrosine phosphorylation-regulated kinase 2 (DYRK2) and casein kinase 1 phosphorylate cytoplasmic NFATs and prevent nuclear translocation, whereas export kinases such as DYRK1 and glycogen synthase kinase 3 phosphorylate nuclear NFATs and promote their export.
- Overexpression and increased transcriptional activity of NFAT isoforms has been detected in various human solid tumours and cell lines, as well as haematological malignancies. This leads to the induction of genes that promote cellular phenotypes that are associated with tumour progression, such as proliferation, survival, migration and invasion phenotypes.
- NFAT isoforms promote the migration and invasion of tumour cells, prerequisites for metastatic dissemination. These phenotypes are mediated by the transcriptional induction of NFAT target genes in tumour cells, such as prostaglandin E2 and lysophosphatidic acid.
- NFATs are directly implicated in promoting tumour angiogenesis. In endothelial cells NFATs are activated by vascular endothelial growth factor A and promote vessel formation by inducing pro-angiogenic genes such as cyclooxygenase 2.
- The activation of NFATs in tumour cells and the tumour microenvironment induces soluble factors that function through both paracrine and autocrine mechanisms to promote tumour progression.
- Inactivation of NFATs decreases tumour formation. This is consistent with pathophysiological settings of increased expression of the calcineurin inhibitor Down syndrome candidate region 1, which attenuates the activation of NFATs and reduces tumour incidence.
- Inhibition of NFAT activation using small-molecule inhibitors is predicted to suppress tumorigenesis. Paradoxically, patients receiving immunosuppressive therapy that blocks NFAT activity have a higher incidence of cancer.
- Future cancer therapy targeting NFATs must take into account the cell type-specific phenotypes associated with deregulated the activation of NFATs.

the RHR region of homology to the calcium-regulated isoforms[11]. NFAT5 does not possess a calcineurin-binding site, and therefore is insensitive to calcium and calcineurin[11,12]. The NHR domain contains the transactivation region of NFATs, which binds promoter elements and so initiates gene transcription. The NHR also contains numerous serine residues that are phosphorylated by distinct protein kinases in resting cells and, as discussed below, reversible phosphorylation of NFATs modulates nuclear and cytoplasmic shuttling and so transcriptional activity.

This Review focuses on the NFAT family members that are expressed in cells that comprise the tumour and its microenvironment. *NFAT1–5* mRNA and protein have been detected in multiple cell types in human solid tumours and cells derived from these tumours, including epithelial cells, endothelial cells of the tumour vasculature and infiltrating immune cells (TABLE 1). Although expression of NFAT isoforms, particularly NFAT3, has been detected in various fibroblast cell lines[13], to date there is no information regarding the endogenous expression of NFAT family members in fibroblasts in the tumour stroma, specifically carcinoma-associated fibroblasts or myofibroblasts.

NFAT transcription factors interact with DNA targets in a versatile manner. NFATs bind DNA as homodimers or heterodimers, and more commonly the DNA binding domain of NFATs can cooperate with DNA binding domains from other transcription factors to elicit high-affinity binding[14]. The best documented example is the unrelated transcription factor activator protein 1 (AP1; comprised of Fos–Jun complexes), which forms a quaternary complex with NFATs and DNA and is the primary transcriptional partner for NFATs required for T cell activation[10,15]. In addition to AP1, NFATs cooperate with numerous other transcription factors that are implicated in cell activation and differentiation, including GATA4, the EGR and MEF2 families and, with particular importance in cancer, forkhead box P3 (FOXP3)[16]. Several recent articles provide a comprehensive review of the mechanisms of gene transcription by NFATs and their binding partners[2,17].

Activation of NFATs by calcium flux

The activation of NFATs by calcium signalling is arguably one of the best characterized mechanisms of signal relay initiated by cell surface receptors. Although it is important to note that the mechanisms of activation of NFATs have largely been deduced from studies in immune cells, the same mechanisms of activation and function of NFATs are recapitulated in cells that comprise the tumour and its microenvironment, particularly endothelial cells. A key rate-limiting event in the activation of NFATs is a rise in intracellular calcium concentrations (which also affects other cancer-associated pathways (BOX 1)). This is initiated by cell surface receptors that stimulate the activation of phospholipase type C (PLC) enzymes such as PLCγ (FIG. 2). Efficient activation of NFATs also requires a sustained calcium signal, and this is achieved by the opening of calcium release-activated calcium (CRAC) channels at the plasma membrane. This occurs in response to PLC-initiated emptying of calcium from the endoplasmic reticulum (ER). In turn, calcium release is sensed by the high-affinity ER calcium sensor stromal interaction molecule 1 (STIM1)[18,19], leading to a conformational change in the CRAC channel protein ORAI1 (REFS 20–23), opening of the channel and an influx of extracellular calcium[24]. Subsequent to the sustained flux through the ER and store-operated calcium channels, calcium binds to calmodulin, which in turn binds to and activates the serine/threonine phosphatase calcineurin. Activation of calcineurin is rate-limiting for NFAT activation in all cells.

In the basal state, NFATs are localized to the cytoplasm in an inactive conformation. More than 20 distinct phosphorylation sites have been identified in NFAT1, 18 of which are located in the regulatory region[25]. These sites are found in multiple distinct serine-rich sequences: the serine-rich region (SRR1) and the SPXX (in which X denotes any amino acid) motifs SP1, SP2 and SP3 (FIG. 1). Dephosphorylation of the SP motifs by calcineurin exposes a nuclear localization sequence and masks a nuclear export sequence, therefore promoting nuclear import leading to transcriptional activation.

Priming kinase
A serine/threonine kinase (such as a casein kinase) that, by phosphorylating specific residues in proteins, enables the subsequent phosphorylation of additional residues downstream of the priming sites, typically mediated by distinct kinases.

Sumoylation
Similar to ubiquitylation in that proteins are post-translationally modified with small ubiquitin-like modifier (SUMO). Unlike ubiquitylation, sumoylation does not target proteins for degradation, instead it facilitates nuclear–cytoplasmic shuttling, transcription and cell cycle progression.

Table 1 | **Expression of NFATs in cells that comprise the tumour and its microenvironment**

NFAT protein	Other names	Expression in immune cells	Expression in tumour cells	Expression in endothelial cells	Expression in CAFs
NFAT1	NFATc2 NFATp	Yes	Yes	Yes	Not determined
NFAT2	NFATc1 NFATc	Yes	Yes	In LECs only	Not determined
NFAT3	NFATc4	No	Yes	Yes	Not determined
NFAT4	NFATc3 NFATx	Yes	Yes	Yes	Not determined
NFAT5	TonEBP OREBP	Yes	Yes	Not determined	Not determined

CAFs, carcinoma-associated fibroblasts; LEC, lymphatic endothelial cell; NFAT, nuclear factor of activated T cells.

Calcineurin also maintains NFATs in a dephosphorylated state in the nucleus[26]. Nuclear export of NFATs is crucial as it leads to the termination of transcriptional activity. Cytoplasmic accumulation of NFATs is achieved by several redundant mechanisms, including the inhibition of calcineurin activity, which can retain cytoplasmic NFATs or promote nuclear export of NFATs. In addition, nuclear kinases rephosphorylate NFATs, leading to export and cytoplasmic retention.

Numerous serine/threonine protein kinases have been identified as regulators of NFAT activity and are subdivided into maintenance and export kinases (FIG. 2), which function to keep NFATs in the cytoplasm by retaining them or promoting their export from the nucleus, respectively. Export kinases include glycogen synthase kinase 3 (GSK3), which phosphorylates the SP2 and SP3 motifs of NFAT1 and NFAT2 — an event that requires prior phosphorylation of NFATs by the priming kinase protein kinase A (PKA)[27,28]. GSK3 is a constitutively active kinase that is phosphorylated and inactivated by PI3K and Akt signalling, which is one of the most frequently deregulated pathways in human tumours[29]. As discussed later, this provides a point of crosstalk between PI3K and NFAT signalling, which is predicted to have important consequences for tumorigenesis. Casein kinase 1 (CK1) functions as both an export and a maintenance NFAT kinase and phosphorylates the SRR1 region[30,31]. MAPK pathways are also frequently hyperactive in human cancers, and it is noteworthy that Jun N-terminal kinase (JNK) and p38 MAPKs phosphorylate the SRR region of NFAT2 and NFAT1, respectively[32,33].

A distinct class of maintenance and export kinases was recently revealed when the dual specificity tyrosine phosphorylation-regulated kinases (DYRKs) emerged from a small interfering RNA screen in *Drosophila melanogaster* as modifiers of the subcellular localization of NFATs[34]. DYRK1 and DYRK2 phosphorylate NFAT1 on the SP3 motif, thereby priming the subsequent phosphorylation of the SP2 and SRR1 motifs by CK1 and GSK3 (REFS 34,35). DYRK1 functions as an NFAT export kinase, whereas DYRK2 phosphorylates NFATs in the cytoplasm and functions as a maintenance kinase (FIG. 2). Therefore, several distinct NFAT kinases maintain the precise subcellular localization of NFATs and, in turn, their transcriptional activity. Although there is now ample evidence that NFAT family members are targeted by distinct maintenance and export kinases, it is likely that additional kinases able to regulate the subcellular localization of NFATs have yet to be identified. Identification of the protein kinases that control activation of NFATs and that are frequently deregulated in human carcinoma may provide important information and possible new therapeutic targets.

In addition to phosphorylation, distinct post-translational modifications have been reported for NFAT family members. Sumoylation of NFAT1 and NFAT2 isoforms provides a separate mechanism of cytoplasmic–nuclear trafficking as it results in their nuclear retention[36,37]. Moreover, NFAT1 is also ubiquitylated by the E3 ubiquitin ligase MDM2 downstream of Akt and GSK3 signalling in breast cancer cells[38,39]. Whether all NFAT isoforms are modified by ubiquitylation and subsequent degradation by the proteasome remains to be determined. Taken together, the sensitivity to calcium flux, the existence of several NFAT kinases and the diverse post-translational modifications demonstrate the complexity of the mechanisms leading to the activation of NFATs.

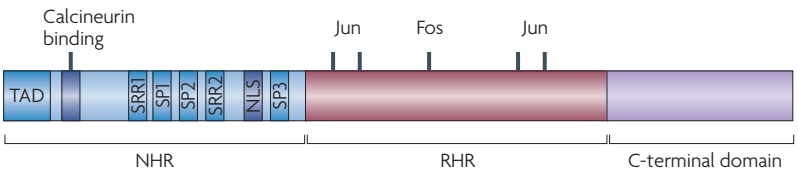

Figure 1 | **Primary structure of NFAT.** The domains depicted are retained in nuclear factor of activated T cells (NFAT) isoforms 1–4, but in NFAT5 the NFAT-homology region (NHR) is truncated and lacks the calcineurin-binding site. The structure shows the NHR region that comprises the amino-terminal transactivation domain (TAD), the calcineurin-binding site, the nuclear localization sequence (NLS) and the serine-rich regions SRR1 and SRR2, as well as the SP1, SP2 and SP3 motifs (Ser-Pro rich) that are targeted by maintenance and export kinases. The Rel-homology region (RHR) comprises the DNA binding motif and points of contact with transcriptional binding partners such as Fos and Jun. Note that NFAT1–4 differ in the size of the carboxyl-terminal domain, and alternative splice variants of NFAT1 (isoforms A, B and C), NFAT2 (isoforms A, B and C) and NFAT4 (isoforms 1, 2, 3 and 4) also exist that differ in the length of the C terminus.

NFAT signalling in tumour cell transformation

Mice with knockout of a single NFAT isoform (BOX 2) indicate that there is a broad range of targets for these transcription factors and suggest redundancy, but also

EMT
A complex process in which genetic and epigenetic events lead to epithelial cells acquiring a mesenchymal architecture concomitant with increased cell motility. Typically associated with the loss of E-cadherin expression.

Hemidesmosomes
Rivet-like structures found in epithelial cells and also keratinocytes that attach these cells to the extracellular matrix. In epithelial cells, hemidesmosomes couple to integrins.

show that isoform-specific differences exist among NFAT1–4. This obviously hampers the use of single or double NFAT-knockout adult mice for cancer-related studies, as a lack of an observable phenotype in whole-animal single knockouts could be due to redundancy, whereas the profound immunological disorders in double knockout mice limits the analysis of tumour progression in adult animals. Therefore, studies on the role of NFAT transcription factors in cancer progression have so far been largely restricted to *in vitro* or cell-based assays. However, isoform-specific functions of distinct NFAT family members in proliferation and tumorigenesis have been identified.

One of the first studies implicating NFATs in proliferation was in fibroblasts in which constitutively active NFAT2 induced cell transformation and colony formation[40]. Similarly, proliferation and anchorage-independent growth of pancreatic tumour cells is dependent on calcineurin activity and NFAT2, which induces *MYC* transcription; this is consistent with the high levels of nuclear NFAT2 in pancreatic cancers[41]. More recently, distinct and opposing roles for NFAT1 and NFAT2 in tumorigenesis were revealed in which NFAT1 functions as a tumour suppressor and NFAT2 as an oncogene[42]. Although NFAT1 and NFAT2 are 72% identical in the C-terminal DNA binding domain, the functional differences between these two isoforms lie in this region. In fibroblasts, constitutively active NFAT1 induced cell cycle arrest and apoptosis, and inhibited HRASV12-induced transformation, whereas constitutively active NFAT2 increased proliferation and transformation. Similar findings were observed in mice; *Nfat1*-null mice were more susceptible to chemically induced carcinogenesis than wild-type mice[42]. These observations underscore the idea that NFAT1 and NFAT2 probably induce a non-overlapping subset of transcriptional targets that suppress and promote cell growth, respectively.

NFATs are also implicated in the induction and progression of haematological malignancies. Active nuclear NFAT2 is found in cases of Burkitt's lymphoma, diffuse large B cell lymphoma and aggressive T cell lymphoma[5,43,44]. In experimental settings of T cell acute lymphoblastic leukaemia (T-ALL), NFAT activation is calcineurin-dependent and pharmacological inhibition of calcineurin reverses cell growth and induces apoptosis[5,44]. Consistent with these findings, the inhibition of calcineurin causes disease regression in mouse models of leukaemia[5]. Considering the major role of the calcineurin–NFAT axis in immune cell signalling, these findings are not surprising. What have remained elusive, however, are the genetic and epigenetic mechanisms that drive constitutive nuclear localization of NFATs, as well as the genes that drive these malignancies.

NFATs also have an important role in maintaining the balance between quiescence and proliferation in stem cells[45]. In stem cells undergoing a transition to a proliferative state, NFAT2 functions downstream of bone morphogenetic protein 4 (BMP4) as a transcriptional repressor of cyclin-dependent kinase 4 (CDK4, which is required for cell cycle progression), and so maintains a state of quiescence in the stem cell population. This may have fundamental consequences for tumour progression, as metastatic tumour cells undergo the epithelial to mesenchymal transition (EMT) and gain the properties of stem cells, providing metastatic tumour cells with self-renewal capacity[46].

NFATs modulate epithelial cell invasive migration

Acquisition of motile and invasive properties is concomitant with the mesenchymal phenotype of tumour cells subsequent to the EMT[47]. Recent studies point to an important role for NFATs in modulating invasive migration, particularly in breast cancer (FIG. 3). Expression of active NFAT1 promotes the migration and invasion of breast cancer cells through Matrigel *in vitro*, whereas expression of NFAT5 promotes migration but not invasion[4]. This is again suggestive of NFAT isoform-specific differences that are probably due to the induction of a non-overlapping subset of genes. In human breast epithelial cells, the non-canonical Wnt ligand WNT5A, which is known to suppress metastatic progression[48], blocks NFAT activation coincident with attenuated migration by a mechanism that partly depends on the binding of CK1 (REF. 49). Conversely, increased expression of NFAT1 and NFAT5 is observed in invasive human ductal breast carcinoma cell lines and also in patients with invasive breast cancer, and this correlates with the expression of the α6β4 integrin, which is released from hemidesmosomes in carcinoma and associates with the actin cytoskeleton. This is consistent with the increased expression of α6β4 that is detected in patients with advanced breast cancer[50,51]. A high proportion of

Box 1 | **Calcium signalling in cancer**

Given the importance of calcium signalling to the activation of nuclear factor of activated T cells (NFAT) in immune cells, any role for NFATs in cancer progression will also be affected by calcium flux. Consistent with this, calcium signalling affects tumour cell proliferation and invasive migration. In migrating immune cells that infiltrate the tumour microenvironment, calcium signalling controls cell polarity, cytoskeletal remodelling and direction[94]. In macrophages, intracellular calcium exhibits a back-to-front concentration gradient, with the lowest concentrations at the front of the migrating cell. This gradient seems to be the opposite of what is expected, as leading-edge lamellae have numerous signalling components that require high calcium levels for signal relay[95]. High calcium microdomains (known as flickers) are most active at the leading edge and therefore facilitate the turning of migrating cells[96]. This may explain the paradox mentioned above: calcium flickers in a low calcium background would allow the correct spatio-temporal activation of calcium-regulated signalling that is required for cell migration[96]. Knockdown of the store-operated calcium release-activated calcium (CRAC) channels ORAI1 and stromal interaction molecule 1 (STIM1) in breast cancer cells attenuates migration *in vitro* and reduces tumour metastasis in mice, also supporting a role for calcium influx in cell migration[97].

Calcium signalling also controls key aspects of cell death by apoptosis, necrosis or autophagy. Overload of calcium in mitochondria affects mitochondrial integrity, which in turn promotes apoptosis. By contrast, changes in calcium flux can also promote the survival of cancer cells, whereby phosphorylation of inositol-1,4,5-triphosphate receptors by the PI3K–Akt pathway results in reduced calcium release and in turn reduced sensitivity to pro-apoptotic signals[98].

The specific contribution of NFATs as effectors of calcium in the cell migration phenotypes described above remains to be established. Regardless, alterations in calcium flux in the tumour microenvironment affect multiple cellular responses, and this is probably exploited by tumour cells, which commonly exhibit changes in calcium flux.

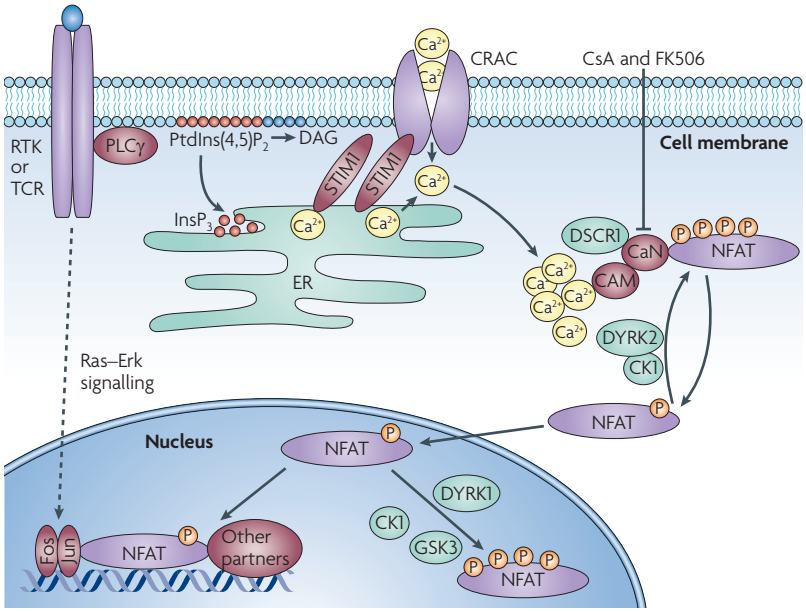

Figure 2 | **Calcium signalling and activation of NFATs.** Receptor tyrosine kinases (RTKs) and immunoreceptors such as the T cell receptor (TCR) activate phospholipase Cγ (PLCγ), which hydrolyses phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to release inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ and loss of calcium binding on stromal interaction molecule 1 (STIM1) induces calcium release from the endoplasmic reticulum (ER). Calcium release-activated calcium (CRAC) channels, including ORAI1, are then opened, allowing a sustained influx of extracellular calcium. Calmodulin (CAM) binds calcium and in turn the phosphatase calcineurin (CaN). Binding of calcium to the calcineurin regulatory B subunit exposes the calmodulin-binding site on the catalytic A subunit. An autoinhibitory sequence in calcineurin is then released from the catalytic pocket, and the phosphatase can dephosphorylate cytoplasmic nuclear factor of activated T cells (NFAT). Inactive NFATs are basally hyperphosphorylated; dephosphorylation promotes nuclear translocation and gene transcription. NFATs cooperate with many other transcription factors, including the activator protein 1 (AP1) complex (Fos–Jun dimers). RTK and TCR activation also stimulates signalling through the Erk pathway, leading to AP1 activation (the dashed line represents the Erk signalling pathway, for which all components are not depicted). The NFAT activation cycle is maintained through complex mechanisms of maintenance kinases that retain cytoplasmic hyperphosphorylated NFATs, such as casein kinase 1 (CK1) and dual-specificity tyrosine phosphorylation-regulated kinase 2 (DYRK2), as well as nuclear export kinases such as CK1, DYRK1 and glycogen synthase kinase 3 (GSK3). These kinases are counteracted by negative regulators of calcineurin, such as Down syndrome candidate region 1 (DSCR1). Pharmacological antagonists of calcineurin, such as FK506 and cyclosporin A (CsA) are potent inhibitors of NFAT dephosphorylation and nuclear accumulation. P, phosphorylation.

patients with breast cancer harbour activating mutations in *PIK3CA*, the gene that encodes the catalytic subunit of PI3K[29]. Downstream of PI3K, the AKT1 serine/threonine kinase attenuates NFAT activity by a mechanism that partly depends on the ubiquitylation of NFATs by MDM2 (REF. 38). The net effect is a reduction of the invasive migration of breast cancer cells, such that a gain-of-function signal (the activation of AKT1) results in a loss-of-function downstream event (NFAT ubiquitylation and degradation) and loss-of-function phenotype (the inhibition of invasion).

The mechanism by which NFATs function as pro-invasion transcription factors probably lies with the transcriptional programme of genes that are induced in tumour cells. NFATs induce the transcription of cyclooxygenase 2 (*COX2*; also known as *PTGS2*) in breast epithelial cells and this is required for the ability of NFATs to promote invasive migration[52]. COX2 catalyses the synthesis of prostaglandins such as prostaglandin E2 (PGE_2). Breast cancer cells have a reduced invasion capacity following knockdown of COX2 or treatment with COX inhibitors such as non-steroidal anti-inflammatory drugs, whereas increased expression of COX2 or the addition of PGE_2 to cells increases cell invasion[52]. This suggests that PGE_2 might function in a cell-autonomous or paracrine manner to influence the invasive migration of epithelial cells (FIG. 3).

NFATs also induce the transcription of the gene encoding autotaxin, exonucleotide pyrophosphatase and phosphodiesterase 2 (*ENPP2*), in breast epithelial cells[53]. Autotaxin is a secreted protein that converts lysophosphatidylcholine into lysophosphatidic acid (LPA). LPA is a potent mitogen and motogen for breast cancer cells[54] (FIG. 3). Autotaxin is highly upregulated in an NFAT-dependent manner[53] in cells expressing the α6β4 integrin. Importantly, autotaxin is also significantly upregulated in breast cancer metastases[55]. Transgenic mice expressing autotaxin or the receptors for LPA (LPAR; also known as the endothelial differentiation gene (Edg) family) in the mammary epithelium have a high frequency of invasive and metastatic carcinoma[56]. Similarly, LPA analogues that function as antagonists for autotaxin and LPARs reduce breast cancer cell migration *in vitro* and significantly inhibit tumour burden in breast cancer xenografts in mice[57]. Because PGE_2 and LPA are secreted molecules, they can function in a cell-autonomous manner by directly binding to the cell surface receptors of epithelial cells and so promote cell migration, presumably by engaging cell signalling pathways that elicit remodelling of the actin cytoskeleton, a prerequisite for cell motility (FIG. 3).

One would also expect that for NFATs to function as pro-invasion transcription factors, they would induce the transcription and secretion of matrix metalloproteinases (MMPs) that are required for efficient basement membrane proteolysis during tumour invasion and metastasis[58]. Although there is some evidence that NFATs are required for MMP induction in myocytes and mesangial cells, to date no studies have addressed MMP regulation by calcineurin–NFAT signalling in carcinoma progression. Similarly, secreted factors that are released in the tumour and its microenvironment are also likely to function in a paracrine manner, particularly COX2 and PGE_2, which have a profound influence on endothelial cell growth leading to angiogenesis.

Regulation of tumour angiogenesis by NFATs

In addition to providing solid tumours with the nutrients and oxygen necessary for tumour cells to survive and proliferate, the vasculature also provides tumour cells with a mechanism to disseminate and metastasize to distant organs such as lungs, liver, bone and brain. As first recognized in mice lacking both *Nfat3* and *Nfat4* (BOX 2), calcineurin–NFAT signalling is essential for angiogenesis and the formation of an intact

Haemangioma
A benign self-involuting mass of proliferating endothelial cells that typically presents in children.

VEGFR
A receptor tyrosine kinase on the surface of endothelial cells that activates downstream signalling pathways subsequent to binding VEGF. VEGFR1 is also known as FLT-1, and VEGFR2 is also known as KDR and FLK1.

Chemokines
A family of secreted cytokines that regulate both immune surveillance and inflammatory responses on infection.

vasculature during development. It is therefore not surprising that NFAT signalling also profoundly affects tumour angiogenesis in humans. Angiogenesis involves the organization and subsequent branching of endothelial cells, as well as the recruitment of vascular smooth muscle cells — a process that requires cell proliferation, migration and differentiation[59]. A prerequisite for tumour angiogenesis is the stimulation of endothelial cell proliferation that is partly achieved by vascular endothelial growth factor A (VEGFA), which is also an endothelial cell permeability factor[60]. As with most mitogens, VEGFA stimulates receptor-mediated activation of PLCγ, leading to an increase in intracellular calcium, calcineurin activation and NFAT nuclear translocation[61] (FIG. 4). In turn, this leads to the transactivation of genes that are essential for angiogenesis, such as *COX2*, resulting in the synthesis of PGE_2, a crucial mediator of tumour cell and endothelial cell migration and tube formation[62]. COX2 has emerged as a key enzyme in the metastatic dissemination of most human tumours, in particular breast cancer cell infiltration to the lungs and brain[63,64]. Activation of NFATs by VEGFA in endothelial cells also induces the transcription of tissue factor (*TF*; also known as *F3*), an important initiator of blood coagulation and angiogenesis[65]. Similarly, NFATs induce granulocyte-macrophage colony-stimulating factor (*GM-CSF*; also known as *CSF2*) in endothelial cells and monocytes, which is important for their differentiation and survival[66].

The function of NFATs in tumour angiogenesis is best illustrated by studies in animal models of cancer progression, as well as the analysis of human pathophysiologies associated with deregulated activation of NFATs. Infantile haemangiomas are rapidly growing areas of disorganized blood vessels that are dependent on VEGFA signalling. Suppressed transcription of a VEGF receptor (VEGFR), VEGF receptor 1 (*VEGFR1*), in these lesions is mediated by the reduced activity of NFAT, and in turn this elicits an increase in VEGFA-dependent VEGFR2 activation, which ultimately causes lesion formation[67]. Normalization of VEGFA or VEGFR2 activity could provide an effective strategy for haemangioma treatment in children who are afflicted with the most aggressive forms of this disease.

Patients with Down's syndrome who reach adulthood have a strikingly lower incidence of cancer than the normal population[68]. This has led to the speculation that one or more of the 231 genes that undergo increased expression as a result of the extra copy of chromosome 21 in Down's syndrome cases might possess tumour suppressor activities. One of these genes is the calcineurin suppressor *DSCR1* (also known as *RCAN1*). *Dcsr1* loss in mice seems to suppress proliferation and increase the apoptosis of endothelial cells[6]. Conversely, increased expression of DCSR1 results in a reduction of tumour angiogenesis through the suppression of activation of NFATs and decreased VEGFA signalling in endothelial cells[69]. This is consistent with an examination of patients with Down's syndrome that reveals increased expression of DSCR1 and the NFAT maintenance kinase DYRK1A, which inactivates NFATs by promoting nuclear export[69]. Similarly, cells overexpressing DCSR1 and DYRK1A have reduced VEGFA-mediated proliferation[69,70]. These findings provide a molecular explanation for the reduced incidence of cancers in patients with Down's syndrome with trisomy of chromosome 21, and underscore the importance of VEGFA and NFAT signalling in tumour progression.

Endothelial cells are also present in lymphatic vessels that are responsible for returning interstitial fluid to the circulation. In breast, lung and gastrointestinal tumours, cells metastasize through lymphatic vessels[71]. It is not clear whether solid tumours promote localized lymphangiogenesis or use pre-existing vessels to metastasize. Regardless, many lymphangiogenic factors, such as VEGFC, influence tumour progression[72,73]. NFAT2 has also been shown to modulate lymphangiogenesis, specifically the patterning process and subsequent valve formation after the initial sprouting of lymphatic endothelial cells[74,75]. Here, NFAT2 functions downstream of VEGFC through interactions with lymphangiogenic promoting factors such as prospero homeobox 1 (PROX1), podoplanin, forkhead box C2 (FOXC2) and VEGFR3. Therefore, NFATs regulate both tumour angiogenesis and lymphangiogenesis.

Chemokines and immune cell infiltration

It is well established that in immune cells NFATs directly induce the transcription of chemokines[24]. Inflammatory chemokines also function as chemoattractants for leukocytes, increasing the migration and recruitment of monocytes and neutrophils to sites of tissue damage[76,77]. Chemokines such as CXCL12 (also known as SDF1α) and CCL21, and their receptors CXCR4 and CCR7, are highly expressed in advanced breast cancer and mediate breast cancer metastasis by promoting the chemotactic and invasive migration of epithelial cells[78,79]. Although presently unknown, any role of NFATs in chemokine signalling and metastatic

Box 2 | **Lessons from NFAT isoform-knockout mice**

The considerable sequence similarity among nuclear factor of activated T cells (NFAT) isoforms, as well as their mode of regulation by calcineurin and nuclear export and maintenance kinases, would suggest a high degree of redundancy. This is emphasized by the relatively mild phenotypes of the mice with knockout of individual NFATs. However, some studies have revealed isoform-specific roles in development. *Nfat1*-null mice have hyperproliferation of splenic B and T cells owing to a lack of Fas ligand expression and therefore escape from cell death[99]. This is concomitant with a reduction in interleukin-4 (IL-4), suggesting that NFAT1 is a positive regulator of cytokine production, consistent with *in vitro* studies. Conversely, *Nfat2*-null mice show defects in heart valve morphogenesis associated with an abnormal cardiac septum, providing evidence for an indispensable role for NFAT2 in cardiac development[100]. *Nfat4*-null mice exhibit abnormal development of myofibres, as well as reduced thymocyte numbers owing to suppression of BCL-2 (REF. 101). Only when more than one NFAT isoform is eliminated are more pronounced phenotypes evident, particularly in the immune system. Mice lacking both *Nfat1* and *Nfat2* have profound defects in cytokine production and cytolytic activity[102]. Mice lacking both *Nfat3* and *Nfat4* are embryonic lethal owing to defects in angiogenesis characterized by vessel instability and disorganization[103]. Mice lacking both *Nfat1* and *Nfat4* have a profound lymphoproliferative disorder[104].

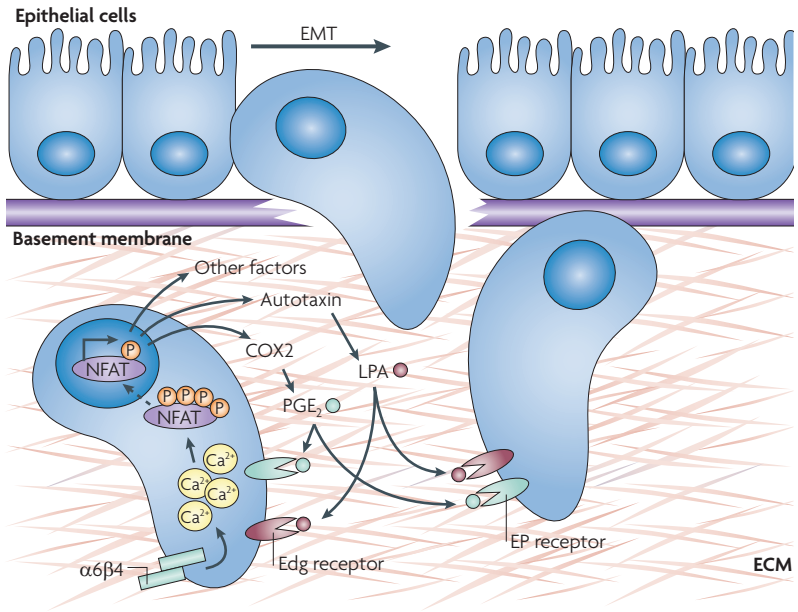

Figure 3 | **NFATs promote tumour cell migration through paracrine and autocrine mechanisms.** Subsequent to genetic and epigenetic deregulation, epithelial cells that reside on the basement membrane undergo a fundamental change in morphology, adopting a mesenchymal architecture, which is concomitant with the acquisition of a motile phenotype. This ordered series of events is collectively termed the epithelial to mesenchymal transition (EMT). Subsequently, the tumour cells degrade the basement membrane and this facilitates cancer invasion into connective tissue that is primarily comprised of extracellular matrix (ECM) proteins. Nuclear factor of activated T cells (NFAT) promotes migration and invasion through multiple non-redundant mechanisms. Engagement of integrins such as α6β4 on tumour cells probably promotes NFAT nuclear translocation through calcium flux. Nuclear NFATs transactivate numerous genes, including those that encode autotaxin and cyclooxygenase 2 (COX2). Autotaxin and COX2 are secreted proteins that catalyse the synthesis of lysophosphatidic acid (LPA) and prostaglandin E2 (PGE_2), respectively. Both LPA and PGE_2 are potent motogens and mitogens that function in both paracrine and autocrine signalling through binding endothelial differentiation gene (Edg) and PGE_2 (EP) receptors, respectively, to promote the invasive migration of tumour cells through the ECM. P, phosphorylation.

dissemination in the tumour microenvironment is likely to be complex. Infiltrating immune cells, as well as mesenchymal stem cells that are localized to breast carcinomas, secrete chemokines that might function in a paracrine manner to influence tumour cell invasion and ultimately metastasis[80] (FIG. 5). However, as NFATs are also active in the tumour cells themselves, chemokines could influence metastasis in a cell-autonomous manner. Regardless, NFAT signalling in the tumour microenvironment is likely to have a significant effect on chemokine signalling. This is underlined by mouse models of leukaemia and lymphoma that reveal hyperactivation of NFATs as a result of paracrine signalling in the tumour microenvironment[5,44]. Similarly, infiltrating macrophages in the tumour microenvironment are directly associated with tumour cells and participate in a paracrine signalling loop between tumour cells that express epidermal growth factor receptor (EGFR) and macrophages that secrete EGF and colony-stimulating factor 1 (CSF1), which in turn promote tumour cell migration[81]. As NFATs induce CSF1 in both monocytes and endothelial cells, the function of this paracrine signalling loop is presumably to promote epithelial cell migration, consistent with the finding that tumour-associated macrophages are located adjacent to the tumour vasculature, the route of metastatic dissemination.

Targeting the NFAT pathway in cancer therapy

NFATs have long been considered ideal targets for therapeutic intervention in immune responses. Two structurally unrelated inhibitors (CsA and FK506) are potent inhibitors of calcineurin–NFAT and are widely used as immunosuppressive agents in tissue and organ transplant to prevent rejection, and also for the treatment of autoimmune disease[82]. Both CsA and FK506 prevent nuclear translocation of NFATs by interfering with calcineurin activation[83]. Specifically, CsA and FK506 bind to the immunophilin proteins cyclophilin A (CyPA) and FKBP12, respectively, and both complexes directly bind calcineurin and inhibit phosphatase activity[84,85]. By interfering with calcineurin activity, CsA and FK506 block the dephosphorylation of numerous substrates in addition to NFATs[86] (FIG. 1). This probably explains the neurotoxicity and nephrotoxicity, as well as the complications from diabetes and high blood pressure, observed in the clinic[87]. Nonetheless, one would predict that as potent calcineurin–NFAT inhibitors, both CsA and FK506 could be effective cancer therapeutics. Somewhat paradoxically, there is actually a significant increase in cancer incidence in patients on long-term immunosuppressive treatments[88]. The explanation for this observation is twofold: first, the increased cancer incidence is probably due to decreased immune surveillance leading to the reactivation of previously quiescent Epstein-Barr virus (EBV)-transformed positive B cells; second, other targets of calcineurin exist that function to modulate phenotypes associated with cancer, such as proliferation and survival. Moreover, systemic administration of CsA and FK506 would affect the entire milieu of the tumour microenvironment, with pleiotropic effects on cellular pathophysiology. It is more reasonable to assume that effective NFAT therapy in cancer will have to come from targeted therapy for the tumour endothelium or the tumour cells themselves, as these are the primary compartments in which attenuation of NFAT activity is predicted to block or even reverse the tumorigenic phenotype.

Because of the caveats associated with CsA and FK506 use, more selective NFAT inhibitors have been developed. A peptide that interferes with the calcineurin–NFAT interaction, termed VIVIT, potently blocks NFAT dephosphorylation and nuclear translocation[89,90], prolongs graft survival in mice[91] and attenuates breast cancer cell invasion[4]. Although the use of peptides as signalling antagonists in therapy is problematic owing to issues with delivery and stability, it will be useful to determine the efficacy of VIVIT peptides in mouse models of carcinoma progression. Small-molecule inhibitors of NFATs hold more promise for therapy, and inhibitors that are similar in structure and function to CsA and FK506, but that exhibit fewer side effects, have been developed. L-732531, an analogue of

Epstein-Barr virus
EBV. Herpesvirus that can transform B cells. It is implicated in the aetiology of several different lymphoid malignancies, such as Burkitt's lymphoma.

FK506, has less kidney toxicity, and similarly ISATX47 is a potent and less toxic analogue of CsA[92,93]. Again, mouse studies are required to determine whether these inhibitors demonstrate any efficacy in preventing or reversing tumorigenesis in mouse models of cancer, beyond their well-documented activities in immune suppression.

As discussed above, DSCR1 is an endogenous inhibitor of the NFAT pathway. In *Dscr1*-null mice, calcineurin is hyperactivated, thereby suppressing cell proliferation and increasing apoptosis[6,69]. A small-molecule strategy targeting the DCSR1–calcineurin interaction could prove to be effective in cancer therapy. Exploiting the interaction between calcineurin and DSCR1 with drugs would in both cases effectively block NFAT activity in the tumour microenvironment, and in turn attenuate the release of factors into the vasculature that promote proliferation and metastatic dissemination. As more information accumulates on the regulation of NFATs in cancer, other strategies are likely to emerge. For example, targeting the NFAT nuclear export kinases such as GSK3 would be predicted to phenocopy inhibition of calcineurin. As GSK3 activity is inhibited by the PI3K–Akt pathway, which is frequently hyperactive in most human solid tumours, this results in diminished GSK3 activity and in turn constitutive nuclear localization of NFATs. Therefore, inhibitors of PI3K or Akt might be expected to reverse GSK3 inhibition, promote export of NFATs and terminate transcriptional activity. Ultimately, any new therapeutic strategy targeting NFATs will have to take into account the pleiotropic functions of NFATs in all cell types in the tumour microenvironment. Moreover, targeted cancer therapy for NFATs will have to take into account the contribution of isoform-specific functions in cancer phenotypes.

Conclusions and future perspectives

In the past few years evidence has accumulated that points to a key role for NFAT transcription factors in cancer progression. Indeed, the function of NFATs is not restricted to the immune system as

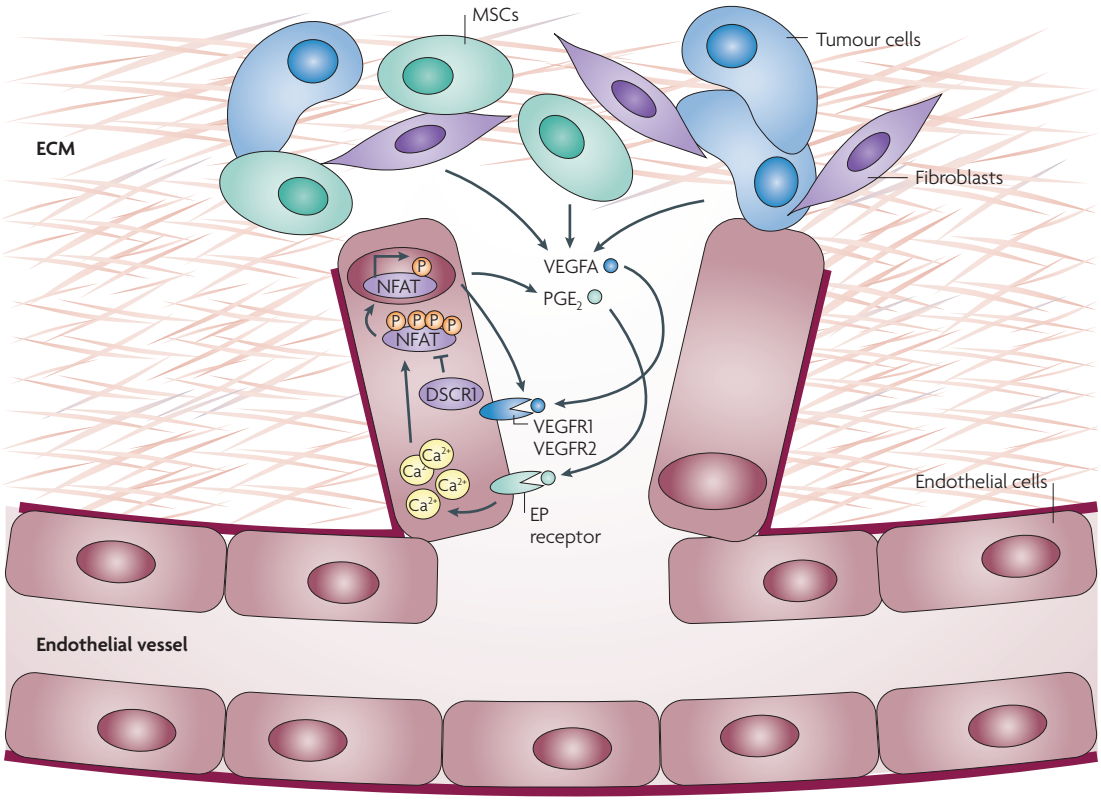

Figure 4 | **NFATs promote tumour angiogenesis.** Vascular endothelial growth factor A (VEGFA) is secreted into the tumour microenvironment by many distinct resident cell types, including endothelial cells, fibroblasts and the tumour cells themselves. If not immediately of use, VEGFA is tethered to the extracellular matrix (ECM). Subsequently, signals that stimulate the angiogenic switch during tumour progression activate mesenchymal stem cells (MSCs), the secretion of matrix metalloproteinases and the release of VEGFA from the ECM. VEGFA then binds VEGFA receptors (VEGFR1 and/or VEGFR2), leading to an increase in intracellular calcium that promotes the nuclear translocation of nuclear factor of activated T cells (NFATs). Activation of NFATs in endothelial cells induces the transcription of *VEGFR1*, which functions in an autocrine loop. NFATs also induce cyclooxygenase 2 (*COX2*) in endothelial cells leading to the synthesis of prostaglandin E2 (PGE_2), which binds to PGE_2 (EP) receptors. Both VEGFA and PGE_2 stimulate endothelial cell proliferation and migration and, ultimately, endothelial vessel formation. Endogenous inhibitors of calcineurin–NFAT, such as Down's syndrome candidate region 1 (DSCR1), block activation of NFATs in endothelial cells and are potent inhibitors of tumour angiogenesis. P, phosphorylation.

Angiogenic switch
The transition of a non-vascularized solid tumour to a highly vascularized state following recruitment of blood vessels.

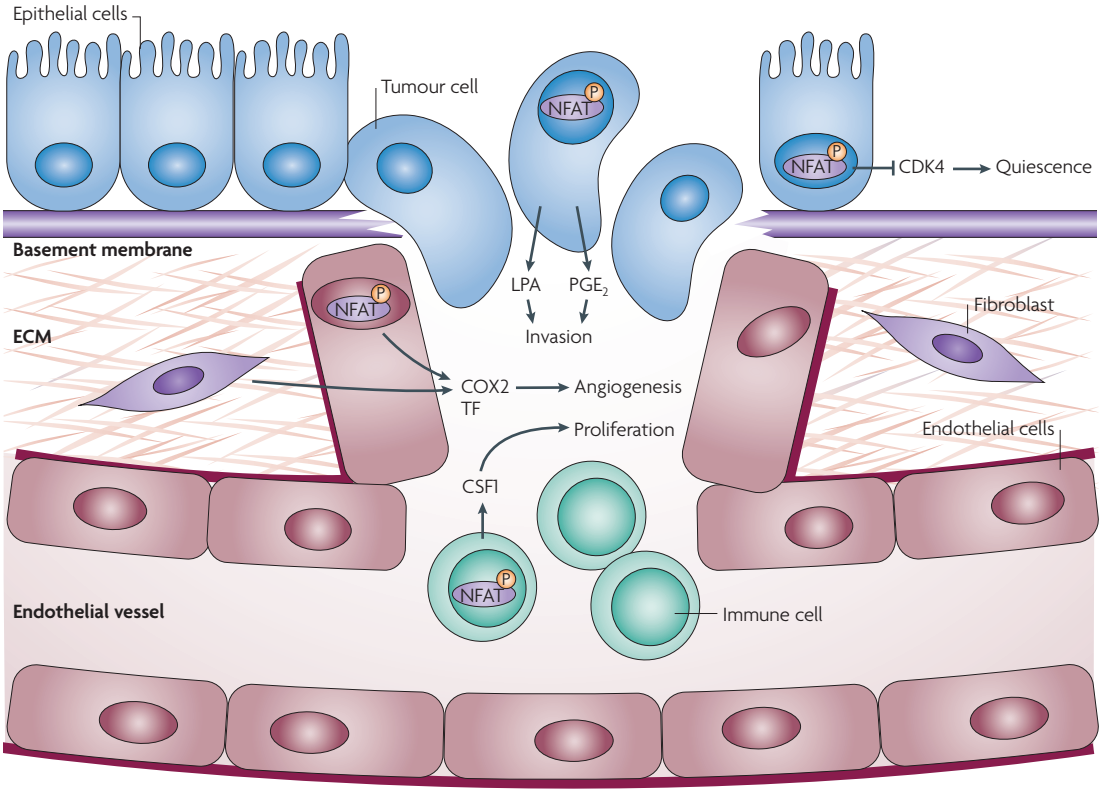

Figure 5 | **Multiple roles for NFATs in the heterotypic interactions of the tumour microenvironment.** Multiple non-redundant mechanisms control the specific roles of nuclear factor of activated T cells (NFAT) transcription factors in tumour progression. In non-tumorigenic epithelial cells, quiescence is achieved by multiple mechanisms, including the NFAT-dependent suppression of cyclin-dependent kinase 4 (CDK4), that are required for proliferation. During tumorigenesis, cells acquire a motile and invasive phenotype that is partly dependent on the upregulation of NFAT activity that in turn stimulates the synthesis and secretion of pro-motility factors such as lysophosphatidic acid (LPA) and prostaglandin E2 (PGE_2). Intravasation of tumour cells into the vasculature is possible only subsequent to the angiogenic switch. NFATs promote angiogenesis through the secretion of pro-angiogenic factors, such as cyclooxygenase 2 (COX2) and tissue factor (TF), by endothelial cells and fibroblasts. Infiltrating immune cells are mobilized to the tumour microenvironment by chemotactic mechanisms that depend on NFAT activation, and in turn secrete local factors such as colony-stimulating factor 1 (CSF1) that promote proliferation. ECM, extracellular matrix; P, phosphorylation.

originally thought: instead these ubiquitously expressed transcription factors control numerous responses in all cells and tissues. A prerequisite for NFAT activation in all cells is nuclear translocation and DNA binding, typically in cooperation with other binding partners. NFAT activity is important for fibroblast proliferation and survival, epithelial tumour cell invasive migration and endothelial cell growth and angiogenesis. However, many questions remain, and the predicted answers to these questions are not only thought to reinforce the idea that the NFAT pathway is a key signalling axis in cancer progression but also might provide new therapeutic avenues for clinical intervention. Most pressing is the development and use of mouse models of cancer in which NFAT isoforms are either deleted or activated in specific cell types or compartments in the microenvironment to evaluate the consequences for tumour initiation and progression. The existence of isoform-specific functions for NFAT family members in phenotypes such as proliferation and tumour suppression has been shown, but the mechanisms responsible for these distinctions have not yet been determined. Similarly, although a handful of proteins such as COX2 and autotaxin have been identified as mediators of the NFAT signal in tumour and endothelial cells, it is likely that numerous other proteins, probably soluble mitogens or motogens, have yet to be described. Genome profiling of tumour cell lines and tumour tissues in which NFATs are active might therefore yield a wealth of new information. It is possible that mutations and/or amplifications in the genes encoding NFAT maintenance and export kinases exist in human cancers, consistent with the reported constitutive NFAT nuclear localization seen in breast cancers. Similarly, mutations or amplifications in the genes encoding NFAT isoforms may exist, although based on recent cancer genome sequencing studies these are not likely to occur at high frequencies. Instead, activation of NFATs in cancer probably occurs by mechanisms that drive or retain NFATs in the nucleus. Answers to these pressing questions may be important for the development of drugs that specifically target NFAT signalling in human cancer.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
COX2 | *DSCR1* | *ENPP2* | *IL-2* | *PIK3CA* | *TF* | *VEGFR1*
National Cancer Institute Drug Dictionary: http://www.cancer.gov/drugdictionary/cyclosporin A | FK506
Pathway Interaction Database: http://pid.nci.nih.gov/canonical pathway | PI3K–Akt pathway
UniProtKB: http://www.uniprot.org
AP1 | CDK4 | DYRK1 | DYRK2 | FOXC2 | FOXP3 | GATA4 | MDM2 | NFAT1 | NFAT2 | NFAT3 | NFAT4 | NFAT5 | ORAI1 | podoplanin | PROX1 | STIM1 | VEGFA | VEGFC | VEGFR2 | VEGFR3

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