7. Vaccinia-related kinase (VRK) signaling in cell and tumor biology

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Abstract. VRK (vaccinia-related kinase) is a group of three proteins in the human kinome. These proteins, mainly VRK1 and VRK2, have been studied in the context of their substrates and interacting proteins in order to identify and characterize their signaling pathway, as well as their effect on other signaling pathways. VRK1 is mostly a nuclear kinase that specifically phosphorylates p53, c-Jun, ATF2, CREB, BAF and histone H3. VRK1 is an early response gene and is implicated in regulation of cell cycle progression. VRK1 is activated in response to DNA damage phosphorylating p53, which is stabilized and activated; this active p53 induces a downregulatory mechanism of VRK1 that permits the reversal of p53 induced effects. The activity of nuclear VRK1 is regulated by its interaction with the Ran small GTPase. Also, VRK1 is a downstream component of the signaling pathway of MEK-Plk3 that induces Golgi fragmentation in mitosis. VRK2

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has two isoforms; VRK2A is cytosolic and bound to endoplasmic reticulum and mitochondrial membranes. VRK2B is a shorter isoform free in cytosol and nucleus. VRK2A affects cellular signaling by interaction with scaffold proteins, as JIP1. The JIP1-VRK2A signalosome blocks the incorporation of JNK, preventing its activation, and thus reducing the stress response to inflammatory cytokines as interleukin-1β and to hypoxia.

1. The vaccinia-related kinase (VRK) family

The completion of the human genome project has led to the identification of 518 proteins that constitute the human kinome [1]. Approximately half of them are not well characterized regarding the signaling pathways in which they are integrated, and their biological functions. But in most likelihood they can probably account for the specificity and regulation of many biological functions. VRK (vaccinia-related kinases) were classified as a new group of Ser-Thr kinases in the human kinome [1], and originally were identified as two human EST, VRK1 and VRK2, which have homology to catalytic region of the B1R kinase of vaccinia virus [2] that is expressed early in viral infection since it is required for viral DNA replication [3-5]. The VRK gene family appeared late in evolution. In lower eukaryotes, *D. melanogaster (NHK-1)* and *C. elegans (Vrk1)*, there is only one member, and no ortholog has been identified in unicellular eukaryotes, like yeasts *S. cerevisiae* or *S. pombe*. In mammals there are three members, two of which (VRK1 and VRK2) are catalytically active [6-8], and a third one, VRK3, is not active and might function as a scaffold protein [9]. The VRK catalytic domain is distantly related to the casein kinase group, forming an early divergent branch in the human kinome [1], but the conservation of the kinase domain is weak and has substitutions in several key residues in this domain [10]. These proteins maintain the structure of the kinase domain, but the rest of the protein is very divergent among them, suggesting that the CK1-like catalytic domain recombined early in evolution with other proteins to form a divergent protein family with new regulatory properties, affecting interactions, regulation, and subcellular localization. In one of these recombination events the kinase domain was picked up by pox viruses (vaccinia, variola, smallpox) and generated the B1R protein, which is the only kinase in poxviruses genome; B1R is required for poxvirus replication and its inactivating mutations can be partially recovered by overexpression of mammalian VRK1, or VRK2 proteins, indicating that in poxvirus there was an additional functional divergence due to viral life requirement [11]. Structurally, the three VRK proteins differ in their regulatory region located C-terminal for VRK1 and VRK2 and N-terminal for VRK3. These regulatory regions are unrelated

among them and have no homology to any known feature identified in other proteins. The length of the regulatory region is variable. It is approximately one hundred amino acids in VRK1 and two hundred aminoacids in VRK2, thus their subcellular localization and regulation is likely to be different among mammalian VRK proteins and those of lower eukaryotes. The relative conservation of catalytic domain of VRK proteins indicates they can have an overlap of potential substrates.

The catalytic domain of these kinases have some key substitutions which make them differ from other closely related kinases, nevertheless two of them VRK1 and VRK2 are catalytically active and have a 56 % sequence identity [6, 8, 12]. These differences might be exploited for the design of VRK specific inhibitors [13], which may be achieved by determination of their crystal structure. The crystal structure of the catalytic domain of VRK2 and VRK3, with 38 % sequence identity and without any significant difference, has been determined providing the framework for future inhibitor development [10].

Human *VRK1* gene is located on the chromosomal region 14q32.2. This gene has a polymorphism, marker rs722869, that in combination with other nine polymorphisms in other genes has proven very useful and is included in the most informative set to study the identification of human continental population structure and genetic ancestry [14]. VRK1 gene expression has been studied in hematopoietic murine development, and its highest expression coincides with the time of maximum cellular expansion in days E11.5 to E13.5 [15]. Endogenous VRK1 protein is detected in different
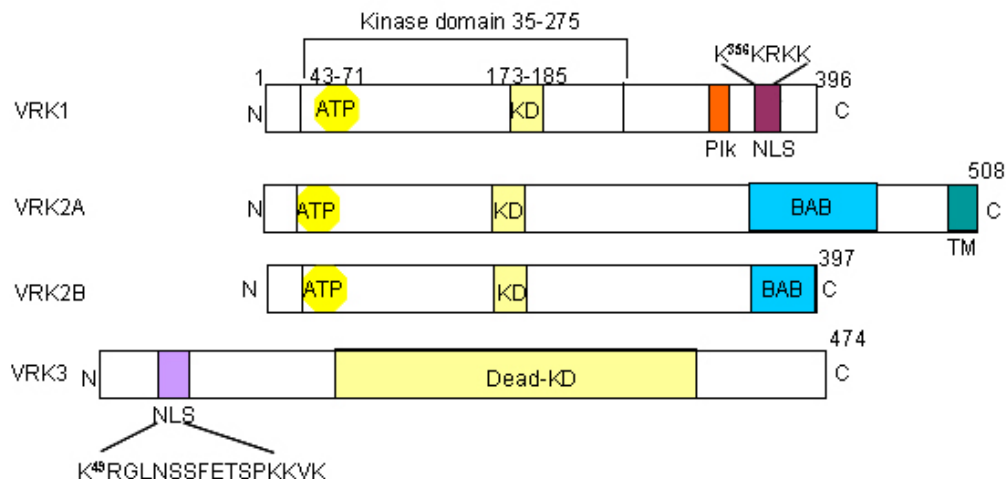

Figure 1. Structure of the three human VRK proteins. ATP: ATP binding site; KD: kinase domain; Plk: consensus target for Plk3. NLS: nuclear localization signal. BAB: Basic-acid-basic region. TM: transmembrane region.

intracellular compartments, suggesting that localization is regulated and that it might play different functional roles depending on its localization. VRK1 has a canonical nuclear localization signal (KKRKK) in residues 356 – 360, which accounts for its nuclear localization [6, 8]. In interphase the endogenous VRK1 has a granular aspect, probably forming aggregates, dispersed within the nucleus, and in mitosis it is dispersed throughout the cell and does not bind to chromatin. In some cell types the endogenous VRK1 protein is mostly cytosolic presenting a particulate appearance, and a minor fraction is nuclear [16], but the underlying cause is unknown. Also there is a subpopulation in the Golgi apparatus where VRK1 colocalizes with giantin where it participates in the control of Golgi fragmentation, and perhaps in VRK1 recycling [17]. This differential distribution is tissue specific [16]. These different locations of human VRK1 are also detected in human normal tissue biopsies. For example in testes and esophagus VRK1 is detected as nuclear with all antibodies. However in small intestine or kidney, a nuclear and a cytosolic subpopulation are detected depending on the antibody used [16]. The factors determining, and regulating, these different subcellular localizations are unknown.

The human *VRK2* gene has 14 exons and can generate two different transcripts by alternative splicing, one lacking exon 13 that contains a termination codon, encoding two proteins of 508 (VRK2A) or 397 (VRK2B) aminoacids respectively. The largest protein is called VRK2A, and the other is VRK2B [12], both are identical up to residue 393 and have a common kinase domain. The VRK2A protein has, at the end of its carboxy terminus, a hydrophobic region of 17 aminoacids that anchors the protein to membranes in endoplasmic reticulum and mitochondria. VRK2A colocalizes with calreticulin and calnexin indicating its presence in the endoplasmic reticulum [12]; it also colocalizes with mitotracker, a lipidic marker of mitochondria [12]. VRK2B, lacking its hydrophobic tail, is free in the cytosol and the nucleus of the cell. The shorter isoform B is more similar in size to VRK1, lacking the membrane anchor sequence, and detected in those cells where VRK1 is mostly cytosolic, suggesting they might have some functional redundancy, but how their expression is coordinated is not known. Therefore, although both proteins might have similar or identical kinase properties, their natural substrates may be different due to their different subcellular localization. Additional variants of VRK2 have been identified by RT-PCR, but the expected corresponding proteins have not been detected and may represent splicing intermediates. Many cell types have been studied for the expression of both isoforms, such as normal B-cells, several lymphoma cell lines and several frequently used carcinoma cell lines such as HeLa, A549, H1299, MCF7, among others. All of them expressed both messages [12]. All

of them express VRK2A protein, but the level of the VRK2B protein was variable, and in some cell lines was not detectable [12].

The human *VRK3* gene is located on 19q13.33, and codes for a protein with a degenerate kinase domain located in the carboxy terminal region that has no kinase activity due to critical aminoacid substitutions [8]. Its crystal structure has revealed important structural differences that make it unable to bind ATP [10]. VRK3 non-functional kinase-dead domain maintains its overall structure so that it can function as scaffold for other proteins [10]. The VRK3 protein is detected in the nucleus and has a bipartite nuclear localization signal [8, 9].

In humans the expression of VRK proteins has been detected in all tissues studied; but not all cells in a tissue express these proteins. The reason for this heterogeneous expression is not yet known, but it may be associated to the proliferation and differentiation state of individual cells. In normal epithelium higher levels are detected near the basal layer, and the intensity of the signal decreases as the cell matures [18].

2. Biological roles of VRK proteins

The biological information available on the three VRK proteins is very limited, and most of it was obtained by studying the human proteins in two main directions. One of them implies the characterization of the signaling pathway in which VRK proteins are implicated, either by substrate identification or by interaction with other proteins. The other is directed at their effect on some biological response, such as hypoxia, interleukin1β or DNA damage. In both situations it is expected that information will expand as these proteins acquire more relevance in the context of tumor biology, and start to attract more attention. In the case of VRK1 some of its targets have been identified, including several transcription factors (Fig. 2), which can serve as starting points to characterize the VRK pathway by itself, or their interaction with other pathways.

In this review we will summarized the different biological processes where VRK proteins have been shown to participate.

3. VRK proteins and cell signaling

3.1. VRK2 downregulates the response to hypoxia or interleukin-1β mediated by JNK

Many biological processes induced by growth factors, stress responses send signals that activate gene expression. These signals are channeled by a

protein complex of mitogen-associated protein kinases (MAPK) which are activated by two additional kinases, MAPKK and MAPKKK, ordered as consecutive steps. MAP kinases represent the core of multiple signaling pathways in response to a variety of stimuli, ranging from growth factors to stress responses. The response implies multiple biological effects such as proliferation, apoptosis, growth arrests and senescence among others [19, 20]. For each of these steps there are multiple kinases, making up multiple combinations that are very likely to determine the specificity of the responses. The three kinases are often anchored on a scaffold protein; the best known are JIP1, of which there are four members, and KSR1 [21]. But there are also other less well known scaffolds. The levels and subcellular localization of scaffold proteins are critical to determine the distribution of a signal among different signaling pathways, and the asymmetry of this signal distribution can determine the final biological effect. These complexes might be further regulated by additional proteins that might interact, activate or inhibit the signal transmission; among these proteins are the two VRK2 isoforms. Therefore signal specificity is likely to be determined by interactions between components of the core signaling pathway with other proteins, most of which have not yet been identified, but is an emerging field. Among these proteins, VRK2A and VRK3 have been shown to interact and regulate MAP kinase signaling in response to different types of stimulation.

3.2. VRK2 downregulates MAP kinase signaling and JNK activation

Many biological processes are controlled by regulation of the activity of mitogen-activated protein kinases which function like a central hub where signals are received and distributed to exert specific roles. MAPK (mitogen-activated protein kinases) are implicated in a large number of cellular responses to growth factors and stress signals of different types. MAPK signals are transmitted by a complex of three consecutive kinases and for each step there are several possibilities. The combination of these kinases contributes to signal specificity. The three consecutive MAP kinases are assembled on scaffold proteins, of which there are several types [21]. These modules permit the organization of specific signal transmission and can be further modulated by specific interactions with other proteins. One of these new regulators is VRK2, which by its interaction with scaffold–MAPK complexes can alter the balance between different pathways responding to a common signal. Signals regulating proliferation are mainly transmitted by the RAF-MEK-ERK proteins assembled on the KSR1 scaffold, while signals implicated in stress responses are channeled by the TAK1-MKK4/7-JNK complex assembled on JIP proteins [21]. The effect of VRK proteins on

signaling by scaffold proteins has been studied using two different experimental systems; one based on the response to stress mediated by the JNK (c-Jun N-terminal kinase) pathway and the other in response to growth factors such as EGF and mediated by the ERK pathway. The best characterized one is the complex assembled on JIP1. JIP1 interacts with JNK and MKK4, or MKK7, as the third and second kinase respectively. However, responses to a common stimulation using this complex may vary depending on cell type, and the reasons for the difference are not known, but are likely to be related to additional proteins which might modulate the signalosome or signaling complex. The JIP1 scaffold protein is usually thought of as a monomer to which different kinase can bind. However, it is known that JIP1 can also be associated to membranes and form large complexes [22]. In our laboratory we have identified that JIP1 form complexes with a size of 1200 kDa, which includes the VRK2A protein [23, 24]. These complexes can incorporate VRK2, and the composition of the complex can be altered if cells are stimulated [23].
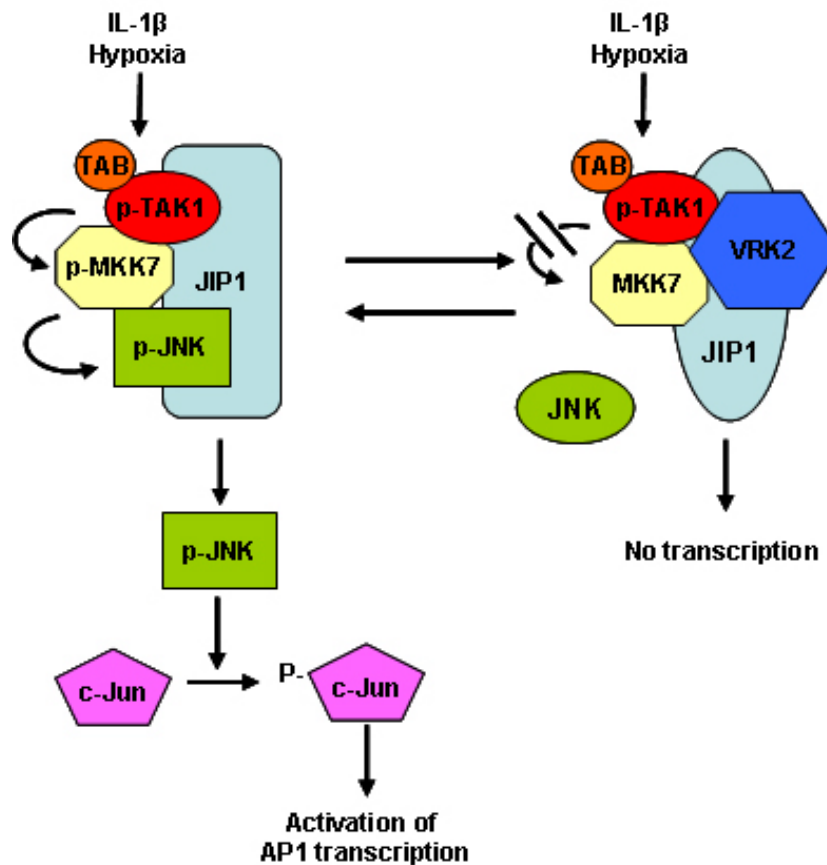

Figure 2. Model of the complexes assembled on the JIP1 scaffold protein which can incorporate VRK2 protein and is modulated by interleukin-1β or hypoxia.

The VRK2A protein subpopulation that is anchored on the endoplasmic reticulum (ER) [12], colocalizes with JIP1 on the ER which suggest that they can interact and modulate MAPK signaling [24]. JIP1 by interacting with ER-anchored VRK2 can be incorporated and form larger complexes; JIP1 up to now has been thought of as a monomer, and by this incorporation in membrane complexes JIP1 can be compartmentalized instead of being free in the cytosol. The formation of these complexes provides the cell with two different signaling alternatives, depending on location and aggregation state, and probably with different substrates. The formation of the signalosome is likely to be assembled on VRK2A protein anchored to membranes, because it is not formed by the VRK2B isoform that is free in the cytosol and nuclei (Fig. 2). The presence of VRK2A is a major requirement to induce the oligomerization state and assembly of the signalosome. In the presence of VRK2A complexes are very large (1000 kDa), but when VRK2A was knocked down the complexes were disassembled, and many of its components were detected as free or forming smaller and incomplete complexes [23, 24]. The effect of VRK2-JIP1 interaction has been studied under two types of stimulation, one in response to the inflammatory interleukin-1β (IL-1 β)[24] and the other in response to hypoxia [23], IL-1 β and hypoxia induce the formation of a large complexes of more than 1000 kDa, known as signalosomes, and containing MAPK and JIP1 protein, and among its components is present the VRK2A [23, 24]. In the complex VRK2A directly interacts with JIP1 and TAK1. The stable interaction of VRK2A with JIP1 can alter the composition of MAPK bound to JIP1. Binding of VRK2A to JIP1 does not affect JIP1 interaction with TAK1 or MKK7, but reduces its binding to JNK, and thus limits the potential activation of c-Jun dependent transcription, which can not be activated because JNK is inactive (Fig. 2).

3.3. VRK3 downregulates ERK activity by interaction with the VHR phosphatase

The nuclear VRK3 protein is able to downregulate ERK signaling by a mechanism different from the one involving VRK2 and MAPK. Because VRK2 is not catalytically active it can only function by mediating protein-protein interactions, where it plays a scaffold role. The effect of VRK3 on MAPK signaling has been characterized by its role in modulating nuclear signals mediated by ERK. ERK (extracellular regulated kinase) is a MAPK that has been implicated in the control of proliferation and cell growth [25, 26]. ERK is the last kinase in the MAPK complex responding to EGF, ERBB2, Ras and Raf, which is most commonly assembled on the KSR1

scaffold. Activation of the pathway by growth factors (EGF) or oncogenes (ERBB2, Ras^{G12V}, or BRAF^{V600E}) induces a phosphorylation of ERK (p-ERK) that has substrates in the cytosol and in the nucleus. Among its cytosolic targets is p90RSK (ribosomal S6 kinase) that activates pleiotropic response associated to cell growth and increase in cell mass [27]. The activated p-ERK is translocated to the nucleus and mainly phosphorylates a large number of transcription factors including ETS, ELK1, Pax6 or c-FOS among others, and their relative levels and activation might condition activation of one type of response or another. One of these pathways is represent by ERK (extracellular-signal regulated kinase). These kinases are regulated by phosphorylation and the level of phosphorylation is control by proteins known as mitogen-activated protein kinase phosphatases (MKPs), the expression or stabilization of MKP activity thus negatively controls ERK activation forming a feed-back regulatory mechanism [28]. In this context, the VRK3 protein, the least known member of the VRK family, appears to control the level of ERK activation [9, 29]. This effect is indirect and mediated by an interaction with the VHR (vaccinia H1-related). In the nucleus VHR dephosphorylates ERK resulting in its inactivation. The nuclear binding of VRK3 to VHR increases its phosphatase activity, and for this process the kinase activity is not necessary, thus this effect is postactivation in the cytosol and therefore its role is to mediate a downregulation, or silencing, the nuclear action of phospho-ERK [9, 29]. In this context, VRK3 functions as a scaffold protein that binds to the VHR phosphatase, which removes the phosphate from an activated MAPK, as is the case of ERK, and thus inhibits or downregulates its nuclear signal.

4. Modulation of transcription factors by VRK proteins

4.1. VRK1 activation of transcription factors can cooperate or be an alternative to MAPK signals

Among the phosphorylation targets of hVRK1 there are transcription factors that are generally regulated by MAPK signaling. Some of these transcription factors can be directly phosphorylated by VRK1 and VRK2B isoform resulting in enhancement of their transcriptional activity, including c-Jun [30], ATF2 [31], CREB [32] and p53 [6]. The nature of these transcription factors reveals potential functional implications for VRK proteins. ATF2, CREB and c-Jun are members of the bZIP family of proteins that can bind to the AP-1 sites to regulate gene expression [33]; this activation is mediated by dimers of these proteins, and depending on the two partners the specificity of the gene selected can be determined.

The c-Jun protein, a component of the AP1 transcription sites, is activated by phosphorylation by JNK (c-Jun N-terminal kinase) in response to stress signals that regulate apoptosis, proliferation and development [34, 35]. The phosphorylation of c-Jun by VRK1 results in the transcriptional activation of Jun independently of the JNK signal. The oncogenic c-Jun can be directly phosphorylated by VRK1 and VRK2B in residues Thr63 and Ser73, which are the same as those phosphorylated by JNK (N-terminal kinase of c-Jun), activating c-Jun dependent transcription [30]. Thus, in situation of suboptimal stimulation, the two kinases might have an additive effect (Fig. 3) and reach maximum c-Jun transcriptional activation [30]. This generates an interesting situation in which c-Jun phosphorylation might be inhibited by the action of cytosolic VRK2A that blocks its activation as a consequence of VRK2A interaction with the JIP1 scaffold protein [24], thus the stress signal response is blocked, as is the case in hypoxia response [23], but at the same time permits its activation by a different route mediated by VRK1, which is regulated in a different way that remains to be identified, but which is associated with entry into cell cycle or responses to DNA damage.
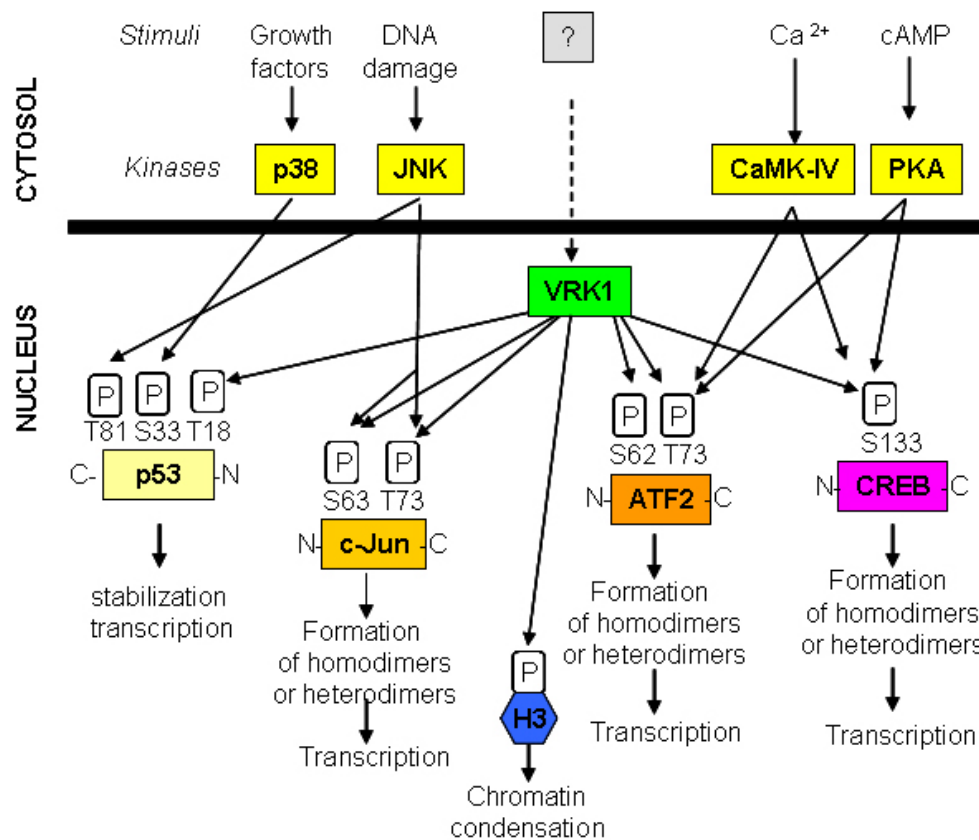

Figure 3. Diagram illustrating the different substrates identified for the human VRK1 kinases and the overlap of its signals with other signaling pathways [6, 30-32].

The ATF2 and CREB transcriptions factors are implicated in pleiotropic responses. VRK1 phosphorylates ATF2 in Ser62 and Thr73 and activates transcription [31]. These two residues are very proximal to those targeted by JNK, Thr69 and Thr71; all within the same region (Fig. 3). These slightly different positions might regulate ATF2 by differential interaction properties with other transcriptional cofactors, and thus affect the specificity of gene transcription. Furthermore, these differences in phosphorylation target can generate a potential cooperative activation by suboptimal stimulation of each kinase, VRK1 and JNK, or respond alternatively to one signaling route or the other. But also the ATF2 residues targeted by VRK1 are the same as those targeted by calmodulin-dependent kinase IV or protein kinase A [31], permitting cooperation between more than one signaling pathways [31]. Thus at least four signaling routes can converge on ATF2 (Fig. 3).

Another target of VRK1 is CREB, a factor identified in response to cAMP. The CREB transcription factor can also be activated by phosphorylation in Ser133 by VRK1, and is required for transcription of cyclinD1 (*CCND1*), VRK1 forms part of the transcriptional complex of this gene in the G1 phase of the cell cycle. This CREB activation was previously known as an effect mediated by a cAMP-response element in CCND1 promoter [32]. Thus two transcription factors responding to cAMP, ATF2 and CREB, are regulated by VRK1, but the functional interaction between VRK1-mediated pathway and cAMP responses are not known.

4.2. Regulation of p53 by VRK1, a novel regulatory loop between VRK1 and p53 in response to DNA damage mediated by DRAM

The tumor suppressor p53 was the first target identified for VRK proteins. VRK proteins can stabilize and regulate p53 by a unique phosphorylation of Thr18. VRK1 [6, 36] and VRK2 [12] phosphorylate p53 specifically is Thr18, a residue that is critical to maintain the loop structure in its N-terminal tansactivation domain (TAD). The stabilization is a consequence of the disruption of the hydrogen bond between Thr18 and Asp21 in p53 necessary to maintain the structure of the loop that binds to Hdm2/Mdm2 [37, 38]. This Thr18 is the critical residue controlling the selection of binding partner by p53 TAD, its phosphorylation increases seven fold its binding to transcriptional coactivators, such as p300 [39], and dephosphorylated p53 interacts preferentially with Hdm2. The effect on p53 interactions is very consistent with the consequences of phosphorylation; when p53 is phosphorylated in Thr18 there is a reduction in ubiquitination and an increase in acetylation, as well as in transcriptional activity [36]. This Thr18 specific phosphorylation changes by three orders of magnitude the

differential binding from Hdm2 to p300 and TAZ1 [39]. Other well-known residues such as Ser15 or Ser20 have a much weaker contribution to selection of binding partner and play a secondary role in this context, although they are the most characterized in response to DNA damage by the ATM/CHK2 [40, 41]or ATR/CHK1 pathways [42].

The stabilization of p53 by VRKs renders a p53 molecule that cannot be degraded and thus accumulates in the cell; a persistent accumulation of p53 will not permit life because it induces either a blockade of cell cycle progression or apoptosis. In addition, the VRK1 protein that functions as a p53 activator is very stable with a half life of four days thus if activated it will maintain p53 in a non-degradable and stabilized form. These characteristics suggested that regulation of VRK proteins level probably requires an active mechanism of protein degradation, which will permit p53 downregulation at the same time. Therefore it is very likely that an autoregulation between p53 and VRK1 must exist. There was an initial observation which suggested that such potential mechanism was functioning because in cell lines that have a high level of p53 there was a lower level of VRK1 compared with cell lines that have a smaller amount of p53, and a higher level of VRK1 [43]. In experimental systems, overexpression of p53 was always followed by a reduction in VRK1 level [43]. The drop in VRK1 protein was not accompanied by a reduction in VRK1 gene expression suggesting it was an indirect effect of p53. Nevertheless, this downregulation was dependent on p53 transcriptional activity, probably by regulating the expression of a not yet identified protein that is the one that targets VRK1 for degradation [43].

The structural requirements of the p53 molecule required to induce VRK downregulation have been identified using different p53 variants containing mutations, deletions or conformational mutants [43]. The N-terminal TAD (trans activation domain) was studied with the p53$^{L22Q/23WS}$ conformational mutant, phosphorylation mutants in all its Ser or Thr residues; none of them affected VRK1 downregulation [43]. The p53 DNA binding domain was studied using the three most common mutations detected in sporadic or hereditary (Li-Fraumeni syndrome) human cancer. The conformational mutant p53^{R175H}, or the p53^{R248W} and p53^{R273H} DNA-contact mutants. All these mutants were unable to induce downregulation of VRK1 levels [43]. Thus a functional p53 DNA binding domain is necessary and supports the requirement of p53 transcriptional dependence of this VRK1 downregulation.

The VRK1 protein is not ubiquitinated by Hdm2, or other ubiquitin ligases, as demonstrated by its insensitivity to either overexpression of mdm2 or to proteasome inhibitors [43], suggesting that the proteasome pathway is not implicated in VRK1 downregulation. Alternatively, downregulation of

VRK1 is sensitive to inhibitors of late-endosome to lysosome transport such as chloroquine [43], and to inhibitors of lysosomal protease activity. These results suggested that p53 induces the expression of a protein that regulates VRK1 and targets it for lysosomal degradation. This protein is DRAM, a lysosomal membrane protein of 238 aminoacids and has six transmembrane domains equally spaced [44]. DRAM expression is dependent on activation of transcription by p53, and expression is lost in case of common p53 mutations in human cancer, such as p53^{R175H}, p53^{R248W} and p53^{R273H}. These requirements are identical as those for VRK1 downregulation induced by p53, thus DRAM is a candidate to be its mediator, and its overexpression of DRAM downregulates VRK1. The induction of DNA damage by ultraviolet light results in accumulation of p53, followed by expression of DRAM and downregulation of VRK1. Thus p53 has a double autoregulatory loop to control its intracellular levels, activated p53 is phosphorylated and induces *HDM2* and *DRAM* gene expression. But hdm2 ca not degrade p53 if it is phosphorylated, thus the removal of VRK1 by its lysosomal degradation mediated by DRAM permits the accumulation of unphosphorylated p53 that is now susceptible to ubiquitination by hdm2 and can be degraded in the proteasome (Fig. 4).
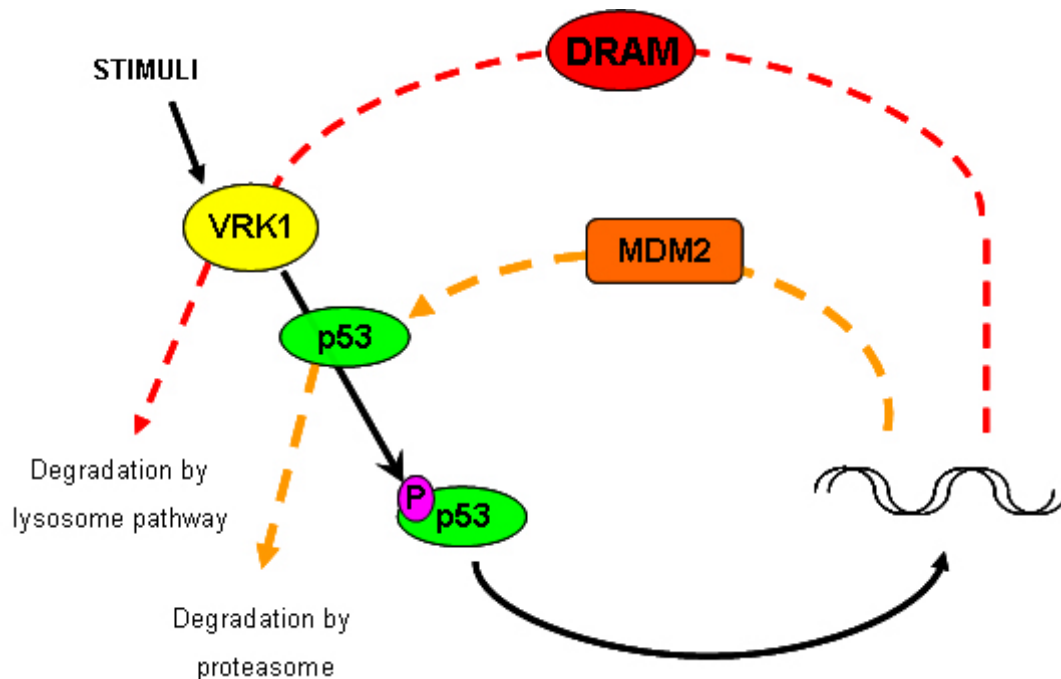

Figure 4. Autoregulatory loop of VRK1 dependent on p53. Phosphorylated p53 activates transcription of Mdm2 that downregulates p53 and of DRAM that downregulates VRK1.

5. VRK proteins in processes required for mitosis

5.1. VRK1 expression and cell cycle

Expression of VRK1 and VRK2 was originally associated to proliferation, because of their expression in proliferating tissues such as testis, thymus, and fetal liver [2] and in the rapid proliferation stage of B-cell development in murine embryos [15].

The expression of human *VRK1* gene is regulated in cell cycle progression. Serum withdrawal results in endogenous *VRK1 gene* silencing in human fibroblast, parallel to the loss of phopho-Rb, and also in loss of expression dependent on the human VRK1 gene promoter coupled to a luciferase reporter [45]. Addition of serum induces expression of *VRK1* RNA at the same time as MYC, *FOS* and *CCND1* (cyclinD1), indicating *VRK1* is an early response gene. Knock-down of endogenous VRK1 results in lack of induction of cyclin D1 and a cell cycle block, early in G1 [36, 45].

In human head and neck squamous cell carcinomas (HNSCC), VRK1 protein is expressed at high levels and positively correlates with several proliferation markers such as Ki67, cyclins B1 and A, cdk2, cdk6 and cdc2. In addition in human lung carcinomas, there were higher levels of VRK1 in those tumors that have a p53 mutation, consistent with an inactivation of the p53 dependent autoregulatory loop [46].

5.2. VRK1, in the control of nuclear membrane formation

The VRK1 and VRK2 proteins, as well as the vaccinia virus B1R kinase, phosphorylate the BAF1 protein (barrier to autointegration factor 1), a 10 kDa protein that forms dimers. The BAF1 protein binds to DNA in a non-sequence specific manner [47], and to proteins containing LEM domains, which are present in the inner nuclear membrane [48]. VRKs and B1R phosphorylate residues Ser4 or Thr2/Thr3 in the N-terminal domain of BAF1. The phosphorylated BAF1 presents a reduction in its binding to DNA and LEM-containing proteins [49]. Phosphorylation of BAF1 in Ser4 delocalizes emerin and interferes with emerin binding to lamin A, both in mitosis and interphase [50]. The overexpression of VRKs reduces BAF1 interaction with nuclear chromatin and results in its dispersal [49]. Nuclear disassembly is a process necessary for cell cycle progression, thus BAF1 phosphorylation by VRK1 might be dependent on functional changes in the activity of VRK1, a kinase that is regulated in cell cycle, and required for G0 exit and G1 progression [45], and its knock down causes a block of cell cycle progression [36, 45]. VRK1 is expressed as the cells enter the cycle from G0 to G1 and

the highest levels are achieved at G1/S transition [18]. The VRK1 protein is very stable and therefore is ready to act when the nuclear envelope has to disintegrate in order to permit chromosome segregation. The *C. elegans Vrk-1* protein, a unique ortholog of human VRK proteins, also phosphorylates the BAF1 protein and affects nuclear membrane structure [51]. But *C. elegans Vrk-1* protein is much larger, 610 aminoacids, completely unrelated to that of human VRK1 protein.. The conservation is restricted to the kinase domain, but there is no relation between their carboxyterminal regions. Thus *C. elegans* and human VRK1 have a common substrate, but probably a different regulation because of their different C-terminal region. Vaccinia virus B1R kinase also has among its substrates the BAF protein that is required in vaccinia life cycle at a time when disintegration of the nuclear envelope takes place, and this function can be partially rescued by overexpressed human VRK1 in virus with B1R mutations [49].

5.3. Regulation of VRK1 activity by interaction with Ran and nuclear dynamics

The enzymatic activity of VRK1 can be regulated by different mechanisms that can affect either its kinase activity or its substrate specificity. These regulatory mechanisms include either protein interactions or covalent modifications. Using a proteomics based approach several interacting proteins have been identified and some of them have already been validated and are now components of emerging VRK signaling pathways.

The first allosteric regulator of VRK proteins is the small GTPase Ran, the only nuclear member of the large Ras GTPase family. Ran stably interacts with the three VRK proteins, but only the Ran interaction with VRK1 has been characterized [52]. Small GTPases have two forms depending on the bound nucleotide; inactive or bound to GDP, and active or bound to GTP. Ran-GDP is mostly located in the cytosol and participates in the nuclear transport mechanism; Ran-GTP, is located in the nucleus, and the nucleotide exchange from Ran-GDP to Ran-GTP is mediated by the action of RCC1 its GEF (Guanine exchange factor) in the nucleus. During mitosis Ran-GTP can form a concentration gradient, with the highest concentration near condensed chromosomes [53]. VRK1 contributes to chromatin condensation by phosphorylation of histone H3 [52, 54]. Ran-GTP binds to VRK1 but does not alter its activity, but VRK1 binding to Ran-GDP inhibits VRK1 kinase activity [52], permitting the formation of a nuclear gradient of VRK1 activity in the nucleus depending on its partner interactions. VRK1 will be activated near the chromosomes, where it can participate in chromatin condensation by

phosphorylation of histone H3 [54], a phosphorylation that is lost by knocked-down of VRK1 [52]. A diagram of the regulation of VRK1 by the Ran small GTPase is shown in Fig. 5.

Also some histones are substrates of VRK proteins. NHK (nucleosomal histone kinase), is the unique VRK ortholog in *Drosophila melanogaster*, and phosphorylates mitotic histones H2A which is required for acetylation of H3 [55-57], a phosphorylation also required for mitotic progression [58]. Human VRK1 is able to phosphorylate histone H3 in Ser10 resulting in chromatin condensation, and cooperating with Aurora B, a process also required for progression of mitosis [54]; this phosphorylation of H3 can be inhibited if human VRK1 interacts with RanGDP [52].
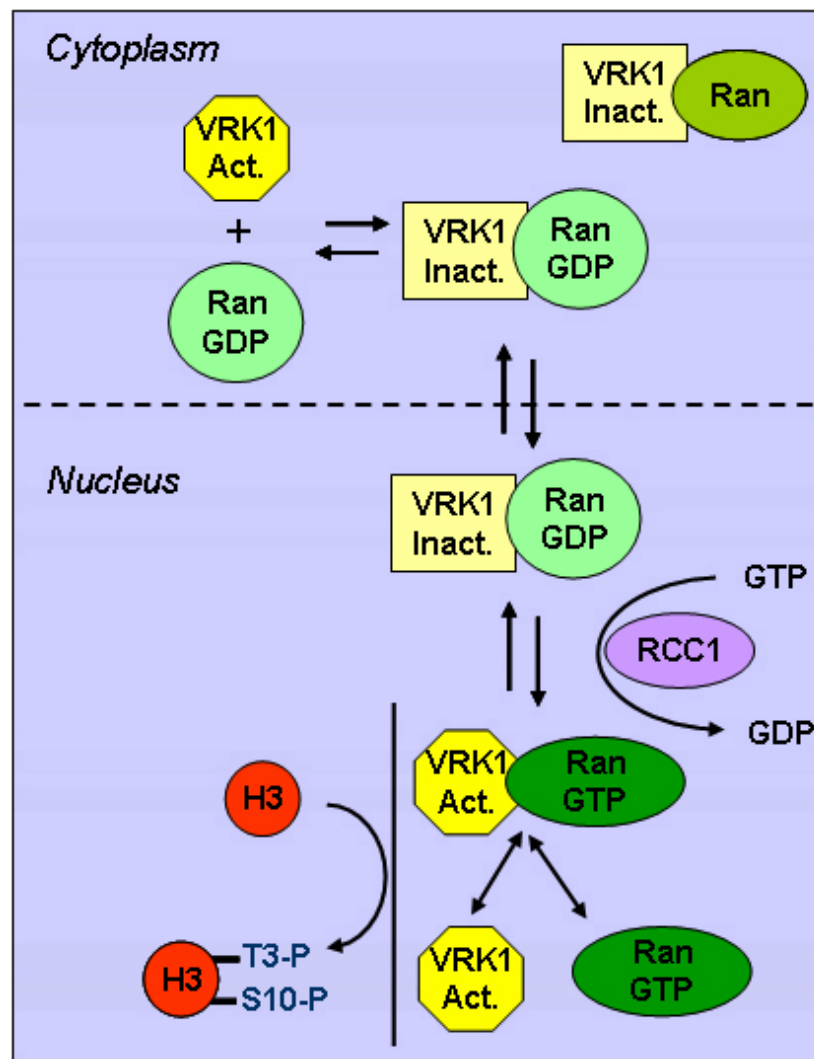

Figure 5. Interaction between VRK1 and Ran. Effect on VRK1 kinase activity depending on the loading state of the small GTPase Ran. [52].

5.5. The MEK-Plk3-VRK1 pathway: Specific phosphorylation of VRK1 in Golgi fragmentation

Golgi fragmentation is a necessary process during mitosis in order to redistribute this organelle into daughter cells. The regulation of this fragmentation is not well known, but mitogenic signals mediated by MAP kinases, particularly MEK1, that induce Golgi fragmentation [59-62], representing an upstream component. A step downstream of MEK1 is mediated by plk3 (polo-like kinase 3) [63], a member of the plk protein family [64]. There is a subpopulation of VRK1, detected with a specific antibody, which is partially located in Golgi colocalizing with markers such as giantin of GM130 [17], which suggests VRK1 might participate in processes regulating Golgi functions. VRK1 participates in this process as a downstream target of Plk3, since VRK1 has a consensus sequence for phosphorylation by Plk proteins. Plk3 phosphorylates VRK1 in Ser340 [17]. Knocking-down VRK1, or the use of a catalytically inactive VRK1^{K179E}, blocks Golgi fragmentation induced by either MEK1 or Plk3 [17]. Thus, one of the cytosolic VRK1 roles is to contribute to Golgi fragmentation in G2/M in mitosis, as a new downstream step of this pathway in mitosis (Fig. 6).
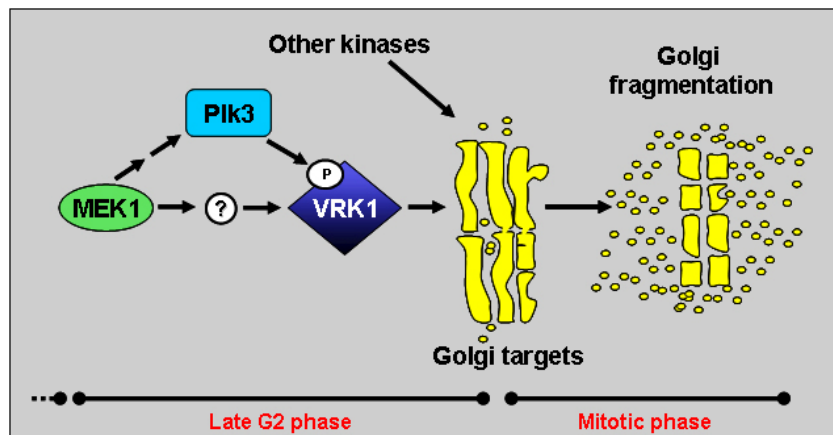

Figure 6. VRK1 is a downstream step in the signaling pathway required for Golgi fragmentation in mitosis [17].

Conclusions

VRK proteins are a group of new human serine-threonine kinases forming a distinctive branch in the human kinome. Among its targets there are a variety of transcription factors implicated in the response to DNA damage, such as p53, or stress responses such as p53 or c-Jun. VRK1

participate in several biological processes including regulation of cell cycle entry and progression, response to DNA damage, nuclear envelope assembly and Golgi fragmentation. VRK2 also modulates signaling by MAPK pathways playing and inhibitory role after its incorporation in a signalosome with scaffold proteins. The VRK signaling pathway is still partially characterized and is expected that its contribution to cell and tumor biology will increase significantly in a near future.

Acknowledgements

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