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MINI-REVIEW



# UnPAKing RUNX3 functions–Both sides of the coin

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**ABSTRACT**
Post translational modifications of RUNX3 have been shown to play an important role in directing RUNX3 functions. In this review we highlight the phosphorylation dependent functions of RUNX3 as regulated by PAK1 and its implications on tumorigenesis.

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The family of RUNX transcription factors are known to have essential roles in the development and cancer,[1,2,3] This family possess 3 members in mammals – RUNX1, RUNX2 and RUNX3 which all share an evolutionarily conserved RUNT domain at their N-terminal end critical in binding to their interacting partners. All RUNX proteins forms a heterodimeric complex with a $\beta$ subunit called CBF-$\beta$ (core binding factor $\beta$) facilitated by RUNT domain which binds to the core DNA sequence 5′ YGYGGT 3′ found in enhancers and promoters of many genes. They are involved in either activation or suppression of the transcription of targeted genes.[4] Even though certain degree of redundancy in their functions is reported despite their strong homology, RUNX proteins have been accounted to execute distinct tissue or cell context functions.[5] RUNX1 has shown to have essential functions in haematopoiesis, and in human leukemia, its gene is involved in chromosomal translocation frequently.[6,7] RUNX2 functions are well implicated in osteogenesis,[8,9] and in cleidocranial dysplasia.[10,11]

## RUNX3 and cancer: Intricacy

In 2002, Yoshiaki Ito and his colleagues reported that RUNX3 has tumor suppressor properties in gastric carcinogenesis and shown to be closely associated with TGF-$\beta$ signaling pathway.[12,13] It was also identified that RUNX3 plays a crucial role in T-cell differentiation,[14,15] and neurogenesis of dorsal ganglia.[16,17] Analysis from the human gastric tumors have shown significant reduction in RUNX3 levels which correlated with cancer progression in gastric tumors. Studies from the RUNX3 knockout mice revealed that RUNX3 loss causes gastric hyperplasia. Reduction or complete loss of RUNX3 in gastric cancers was attributed to either hemizygous deletion or hyper methylation of RUNX3 promoter region.[10] Interestingly, the locus of the RUNX3 gene (1p36) is present in a region which is among the frequently affected regions of most cancers suggesting its role in these cancers.[18,19,20] Tumor suppressor activity of RUNX3 in gastric cancers was strengthened with the observation of decreased tumorigenicity in human gastric cell lines when RUNX3 was re-introduced.[21] Transcriptional repression of RUNX3 resulting from its promoter hyper methylation have been reported in pathogenesis of colorectal cancers.[22,23] RUNX3 is essential in arresting cell proliferation and plays a crucial role in apoptosis functions induced by TGF-$\beta$ signaling pathway in esophageal adenocarcinoma cells.[24]

RUNX3 is shown to have tumor suppressor role in hepatocellular carcinoma. It has been reported that its gene is involved in frequent allelic inactivation.[25] RUNX3 is shown to have pivotal role in patient survival and prognosis in lung adenocarcinoma. Patients with higher RUNX3 expression have shown significant increase in the survival rate.[26] It is also shown that RUNX3 is a critical tumor suppressor in lung cancers.[27,28] Underexpression of RUNX3 reported in breast cancer cell lines and breast cancer tissues is caused by the hyper methylation of its promoter. RUNX3 has shown tumor suppressor behavior in studies conducted on breast cancer cell lines.[29] Frequent inactivation of RUNX3 by promoter hyper methylation and protein mislocalization is reported in oral squamous cell

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carcinoma.[30] RUNX3 plays an essential role in the pathogenesis of melanoma and it also serves as an important prognostic marker.[31]

## Regulation of RUNX3 functions

It is well established that down regulation or inactivation of RUNX3 or its functions contribute to the cancer development in solid tumors.[32] The causes behind RUNX3 functional inactivation could be genetic,[12,33,34] epigenetic,[12,35,36] or cellular modifications post its synthesis.[2,29,37] In many breast cancer cell lines, hemizygous deletion of RUNX3 is observed contributing to the complete loss of the RUNX3 protein.[38] Hyper methylation is often observed in the promoter region of RUNX3 gene in many breast cancer cell lines and breast cancer tissues correlating to its expression levels.[29,38] The recent findings suggest that mislocalization of RUNX3 protein from nucleus to the cytoplasm also contributes to the loss of tumor suppressor functions and in some instances, gain of functions. Mislocalization of RUNX3 is reported in gastric cancer,[37,39] breast cancer,[40] ovarian cancer,[41] and pancreatic cancer.[42] Although the mechanisms by which RUNX3 loss is not clear in genetic and epigenetic modes, it appears that the key mechanism for the cytoplasmic sequestration of RUNX3 is its post-translational modification, by phosphorylation. It has been established that phosphorylation of serine, threonine, or tyrosine residues of RUNX3 controls its cellular functions. Along with mislocalization, phosphorylation of RUNX3 also changes its stability and its interaction with other proteins. But, the mechanisms that are driving the phosphorylation dependent changes in its sub-cellular localization along with its functions still need to be explored.

## Paradoxical role of RUNX3

Initially, the tumor suppressor function of RUNX3 was well established. First it was evident from the studies in gastric cancers;[12] later similar role was identified in breast cancers,[29,38] pancreatic cancers,[43] colon cancers,[22,23] lung cancers,[26,27,28] prostate cancers[44] and so on. Contrasting to this opinion of RUNX3 function, there has been some reports that overexpression of RUNX3 can trigger tumorigenicity.[45] First glimpse of evidence to this paradoxical role of RUNX3 as an oncogene is provided by Salto-Tellez and his colleagues. They reported overexpression of RUNX3 in nucleus from immunohistochemical analysis in basal cell carcinomas.[46] Later, Nevadunsky *et al* in 2009 provided similar results. Their study reported overexpression of RUNX3 in epithelial ovarian cancer. But interestingly their histochemical analysis revealed that RUNX3 is upregulated in cytoplasm. Increased viability is reported in ovarian cancer cells when RUNX3 is re-introduced, whereas reduced proliferation when RUNX3 is depleted.[47] RUNX3 function as an oncogene is also reported in head and neck squamous cell carcinoma where overexpression of RUNX3 is shown to have enhanced cell proliferation, thus increasing progression of malignancy.[48] Surprisingly, adding to its oncogenic potential, RUNX3 is also involved in chemo-resistance in some cancers. It has been shown that RUNX3 ectopic expression supresses adriamycin-induced apoptosis in HNSCC and imatinib-induced apoptosis in CML.[49,50]

Conclusively, it is evident that RUNX3 has dualistic functions of tumor suppressor and oncogene, but it is cell context dependent. The clinical relevance and the ambiguity of its switching between contrasting functions is still an unresolved and interesting question. However, recently there has been a report that serine 209 phosphorylation on RUNX3 by p21 activated kinase 1 switches its functions from a tumor suppressor to an oncogene in pancreatic ductal adenocarcinoma.[42] It has been shown that phosphorylation on RUNX3 would change its subcellular localization. But the mechanism that drives such a switch in its functions from a post-translational modification is still to be addressed. In another recent report, it was discovered that RUNX3 function as tumor suppressor and tumor promoter in pancreatic ductal adenocarcinoma, but the switch in functions is maintained by regulating the balance between proliferation and dissemination in response to gene dosage of DPC4.[51]

## Role of RUNX3 phosphorylation

Post translational modifications play a pivotal role in variety of cellular processes like signaling, proliferation, cell division, gene expression etc. and perturbances in such processes would lead to the cancer. From the time of its discovery, phosphorylation has been recognized as a global regulator of many cellular signaling pathways, and abnormal phosphorylation is implicated in cancer prognosis. Phosphorylation is involved in the control of proliferation, transcriptional regulation, oncogenic kinase signaling and TP53 activity, among other processes which are important in the occurrence of cancer. Phosphorylation on certain proteins are regarded as pharmacologically targetable and multiple approved therapies has been developed for cancer treatment.[52]

Kinases are involved in the amplification of signals that lead to cell proliferation or apoptosis inhibition by activating transcription factors (e.g. NF-$\kappa$-B, AP1, Myc), inhibiting pro-apoptotic molecules like Bad and Bax, or they involve in the deregulation of the cell cycle control.

Consequently, it can be well understandable that kinases are major players in oncogenesis, which uncovers them as putative targets for anti-cancer drug design.[53] Pak1, which belongs to the Pak family, was identified as a protein that interacts with CDC42 and RAC1, which are members of the Rho GTPase family of proteins.[54] These proteins perform specific and distinct cellular functions which are different from the functions of Ras and have been primarily involved in cytoskeletal reorganization and motility and in the reactive oxygen species (ROS) production.[55,56]

The effector functions of GTPase-activated PAKs are proceeded through their kinase activity and arbitrate the signaling events downstream that bring about the physiologic effects of GTPase signaling. In addition to small GTPases, PAK activity could be influenced by other mechanisms. They include - activating (PDK1) and inactivating (PKA) phosphorylation of PAK.[57,58] Redistribution of activated PAKs to the leading edges of motile cells stimulate the motility and invasion and thus indicates that PAKs are downstream effectors for multiple signaling pathways. Hence, the degree of activation and localization of PAKs are dictated by specific upstream signals and in turn PAKs activate other kinases and effectors by phosphorylating them at specific sites or through protein–protein interaction. This mechanism clearly shows how PAKs amplify and propagate the upstream oncogenic signals, barring the negative feedback imposed through some known Pak inhibitors.[59]

The first evidence for the role of Pak1 signaling in oncogenesis was observed when Ras induced transformation of Rat-1 fibroblast cells was inhibited by ectopically expressed p21- binding domain (PBD) of Pak1.[60] Later studies with a Pak1 inactive mutant which does not show kinase activity confirmed that Pak1 kinase activity was necessary for the Ras-induced transformation of fibroblast cells.[61] Further investigations revealed that Pak1 was essential for transformation induced by signaling molecules like Ras, VAV3, CDC42, RAC1 and RAC3.[62,63] Thus, it confirms that Pak1 signaling as a central module in transformations induced by various small GTPases that are stimulated by mitogenic activators.

Increased expression and activity of Pak1 was reported in breast tumors and higher levels of Pak1 protein expression was identified in higher grade tumors samples. Pak1 expression is largely upregulated in human breast tumors matched with breast cancer invasiveness and tumor cyclin D1 expression, which is known to regulated by Pak1.[64] Along with overexpression of Pak1, increased Pak1 kinase activity was also reported in human cancer cells by the increased activity of small GTPases such as RAC3, which occurs in breast tumors.[62] Pak-interacting exchange factor ($\beta$-PIX), a known activator of CDC42/RAC and Pak kinase activity, was also overexpressed in human breast cancer.[65] Hence, to stimulate Pak1-mediated signaling pathways, breast cancer cells utilize different mechanisms which might result in increased metastatic potential.[59]

Various studies pertaining to the role of post-translational modifications in RUNX3 functions indicate that divergent signals related to oncogenic pathways may cause perturbations in RUNX3 functions mainly by cytoplasmic mislocalization of RUNX3 upon phosphorylation. Table 1 summarizes the various kinases involved in RUNX3 phosphorylation and their functional implications in RUNX3 activities. Until some point, it was believed that phosphorylation of RUNX3 is involved only in RUNX3 functional inactivation, but later reports revealed its potential in the oncogenesis. Recently, we found that oncogenic kinase PAK1 phosphorylates RUNX3 at threonine 209 and have shown to cause switch in its dualistic functions. It was identified that T209 is highly conserved among divergent organisms and might be having a significant role in the functional regulation of RUNX3. We have identified that phosphorylation by PAK1 causes RUNX3 to change subcellular localization to cytoplasm. T209 phosphorylated RUNX3 not only failed to induce p21 activation which is critical for its tumor suppressor activity, it has promoted tumorigenesis in pancreatic cancer cell lines and mouse xenograft studies (See Fig. 1 for a model depicting the PAK1 regulated RUNX3 induced transformation). Abundant presence of T209 phosphorylated RUNX3 in various cancer tissues compared with their normal counter parts which is evident from the immunohistochemical studies also suggests that it might be involved in a signaling pathway promoting oncogenesis. Although the mechanism is unclear, it is sure that the non-transcriptional activity of RUNX3 is involved in this paradoxical

**Table 1.** Summary of Kinases involved in RUNX3 phosphorylation and functional significance upon modifications in RUNX3 activities.

| Kinase | Phosphorylating residue in RUNX3 | Functional significance | Reference |
|---|---|---|---|
| Src Kinase | Multiple Tyrosine Residues | Cytoplasmic Mislocalization of RUNX3 | 39 |
| Pim 1 Kinase | Ser/Thr Residues in RUNT domain | Stabilizes RUNX3<br>Alters Sub-cellular localization of RUNX3 | 41 |
| p21 Activated Kinase 1 (PAK1) | Threonine 209 | Cytoplasmic Mislocalization<br>Oncogenic activity of RUNX3 | 42 |
| Aurora Kinase | Threonine 14<br>Threonine 173 | Critical for DNA binding functions of RUNX3 | 67 |

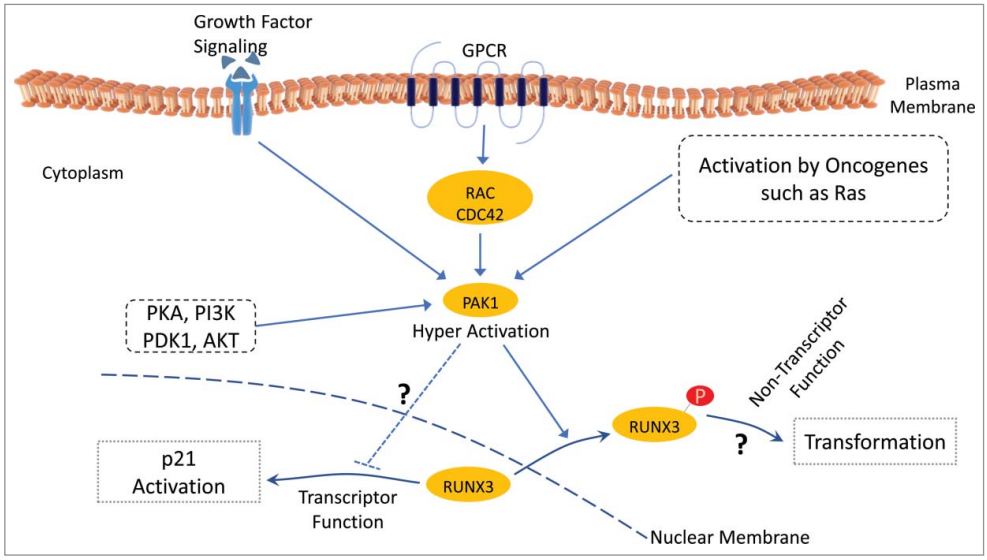


**Figure 1.** A model depicting the possible mode of PAK1 regulated RUNX3 induced transformation. An illustration representing the convergence of multiple signaling pathways involved in the normal activation and hyper activation of PAK1 in normal cells and cancer cells. RAC1 and CDC42 are required for the activation of PAK1 in normal cells. PAK1 also gets activated by PKA, PI3K, PDK1 and AKT signaling pathways. Hyper activation of PAK1 in cancer cells leads to phosphorylation of RUNX3 which in turn switches off the transcriptional role of RUNX3 required for the p21 induction. Phosphorylated RUNX3 is involved in the neoplastic transformation non-transcriptionally by unknown mechanisms.

activity. Previously, it was reported that phosphorylation of serine, threonine or tyrosine residues on RUNX3 by different kinases will cause a change in its stability and activities.[66] Pim-1 kinase phosphorylates 4 serine residues in Runt-domain of RUNX3 and increases half-life of RUNX3 protein. Besides this phosphorylation by Pim-1 kinase significantly increased the cytoplasmic localization of RUNX3 and it has been proposed that RUNX3 behaves as an oncogene when it is mislocalized to the cytoplasm.[41] In another study, it was found that overexpression of Src kinase leads to tyrosine phosphorylation of RUNX3 and its mislocalization to cytoplasm in gastric and breast cancers.[39]

Analysis from breast cancer cell lines and breast cancer tissues revealed that Pin 1 recognizes phosphorylated motif in RUNX3 and causes its proteasome-dependent degradation.[40] However, earlier report showed that phosphorylation of threonine 173 present in Runt domain of RUNX3 by Aurora kinases is essential for mitotic progression and cell cycle arrest and thereby, for its tumor suppressor function.[67,68] Even though, it is quite evident that phosphorylation of Runx3 is associated with its dualistic functions, the precise mechanisms which confer these roles still need to be explored.

In conclusion, based on the recent evidences about RUNX3phosphorylation dependent paradoxical functions, it is imperative that this post-translational modification of RUNX3 probably gives a pharmacological opportunity to intervene and mitigate its functions and thereby, RUNX3 could be a new player in arena of targeted therapy of cancers.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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