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Harnessing the master transcriptional repressor REST to reciprocally regulate neurogenesis

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Abbreviations: REST, repressor element 1 (RE1) silencing transcription factor; NPC, neural progenitor cell; HEK, human embryonic kidney; CTDSP1, C-terminal domain small phosphatase 1; EGF, epidermal growth factor; H-Ras, Harvey rat sarcoma viral oncogene homolog; ERK, extracellular signal-regulated kinase; CK1, casein kinase 1; S, serine; E, glutamate; Pin1, peptidylprolyl cis/trans isomerase; βTrCP, β-transducin repeat containing E3 ubiquitin protein ligase; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; BMP, bone morphogenetic protein; PAX6, Paired box 6; Ngn2, neurogenin 2; Tbr, T-box brain protein; DNA, deoxyribonucleic acid; GTP, guanosine triphosphate; CNS, central nervous system; FDA, The Food and Drug Administration.

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Neurogenesis begins in embryonic development and continues at a reduced rate into adulthood in vertebrate species, yet the signaling cascades regulating this process remain poorly understood. Plasma membrane-initiated signaling cascades regulate neurogenesis via downstream pathways including components of the transcriptional machinery. A nuclear factor that temporally regulates neurogenesis by repressing neuronal differentiation is the repressor element 1 (RE1) silencing transcription (REST) factor. We have recently discovered a regulatory site on REST that serves as a molecular switch for neuronal differentiation. Specifically, C-terminal domain small phosphatase 1, CTDSP1, present in non-neuronal cells, maintains REST activity by dephosphorylating this site. Reciprocally, extracellular signal-regulated kinase, ERK, activated by growth factor signaling in neural progenitors, and peptidylprolyl cis/trans isomerase Pin1, decrease REST activity through phosphorylation-dependent degradation. Our findings further resolve the mechanism for temporal regulation of REST and terminal neuronal differentiation. They also provide new potential therapeutic targets to enhance neuronal regeneration after injury.

New neurons are generated from neural progenitor cells (NPCs) via a process called neurogenesis, which in vertebrates occurs in restricted brain regions.[1] Neurogenesis is associated with biological functions such as learning, memory, and other cognitive functions. Inhibition of neurogenesis via antimitotic agents, radiation, or genetic manipulations has been demonstrated to impair hippocampus-dependent forms of memory in rodents.[2] Studies in songbirds have associated neurogenesis with song learning.[3] Defects in neurogenesis have been linked with many disease states, with cognitive etiologies including developmental disorders (e.g., microcephaly,[4] megalencephaly,[5] and autism)[5] as well as neurodegenerative diseases (e.g., dementia and Alzheimer disease).[6] Ultimately, resolving the signaling mechanisms that regulate neurogenesis is key to advancing our understanding of these biological processes.

Neurogenesis is orchestrated by several signaling pathways originating at the plasma membrane, including Wnt, EGF, FGF, VGEF, and BMP, and terminating in the cell nucleus. These signaling cascades initiate the progressive expression of many transcription factors, including Pax6, Ngn2, Tbr2, NeuroD, and Tbr1. Despite this list of implicated proteins, many gaps remain in our knowledge regarding the signaling mechanisms in the nucleus that leads to transcriptional changes that occur during neuronal differentiation. These nuclear signaling components are attractive targets for treating neurological disorders because they directly regulate cellular differentiation.

Our recent report provides new insight into the signaling mechanisms regulating the repressor element 1 (RE1) silencing transcription factor (REST), a master regulator of neuronal differentiation. REST acts by binding to the DNA chromatin at the RE1 sites near the regulatory regions of neuronal genes to repress their expression.[7] Consistent with its function, REST is present in most non-neuronal tissues including stem cells.[8,9] Many target genes of REST repression have been identified, including those required for the terminally

differentiated neuronal phenotype such as receptors, ion channels, growth factors, and axonal-guidance proteins.[7] Consistent with this role, REST is aggressively degraded in neural stem/progenitor cells,[9] and the clearance of REST from the chromatin allows for the expression of neuronal genes, enabling terminal differentiation.[9]

Prior studies of REST degradation identified a mechanism that involves the phosphorylation of 2 C-terminus sites of REST (E1009/S1013 and S1024A/S1027A/S1030A)[10,11] by casein kinase 1, CK1.[12] Phosphorylation of these 2 sites facilitates binding of a chaperone protein, β-transducin repeat containing E3 ubiquitin protein ligase (βTrCP),[12] which then shuttles REST to the proteasome for degradation.[10,11] However, both CK1 and βTrCP are present in stem cells where REST is abundant, and phosphorylation of residues S1013/1024/1030 does not explain the observed temporal regulation of REST degradation.[9,13]

Our objective was to elucidate the cell signals that are consistent with the timing of REST degradation. Using mass-spectrometry, we identified 14 phosphorylation sites as putative regulatory sites. Cycloheximide degradation assays in HEK cells expressing REST truncations and point mutations revealed that a proline-directed phosphorylation motif at serines 861 and 864 regulates REST stability/activity.[14] Coincidentally, these amino acids were found to be the most frequently phosphorylated.

In light of the previously identified REST degradation sites,[10,11] we investigated the possibility that the mechanisms governing REST degradation were related. Accordingly, we mutated serines 861 and 864 and assessed βTrCP binding at the previously identified down stream REST degradation sites.[10,11] We found mutating serines 861 and 864 indirectly regulated binding of βTrCP to the downstream REST degradation sites.[14] We hypothesized that the mechanism governing βTrCP binding was through either a change in protein-protein interactions or conformation. Consistent with this hypothesis, we identified serines 861 and 864 as a Pin1 binding motif.[14,15] Pin1 is a well-established modulator of protein-protein interactions (including βTrCP),[16] and phosphorylation dependent conformational changes.[15] Importantly, Pin1 is implicated in neuronal differentiation.[16] We found Pin1 recognized phosphorylated serines 861 and 864, and that inhibiting Pin1 activity inhibited the binding of βTrCP.[14] Our data show that serines 861 and 864 shared the same mechanism for regulating REST stability as the previously identified downstream sites.[10,11,14]

Given that Pin1 binding requires a phosphorylated serine or threonine adjacent to a proline,[15] we sought to identify the kinase that phosphorylates these residues. Both serine 861 and 864 are highly predicted for recognition by the extracellular signal-regulated kinases, ERK, 1 and 2. We hypothesized that ERK and its canonical upstream activators, epidermal growth factor (EGF) and the small GTPase Harvey rat sarcoma viral oncogene homolog (H-Ras) would promote phosphorylation at these serines in REST. We demonstrated that serine 861 and 864 are terminal targets of EGF-Ras-ERK signaling and that ERK2 can directly phosphorylate REST.

The EGF-Ras-ERK pathway is implicated in both embryonic and adult neurogenesis.[17,18] In embryonic culture models of neuronal differentiation, EGF treatment correlates with REST degradation.[19] Accordingly, our work demonstrates that inhibiting ERK stabilizes REST. Therefore, our findings implicate phosphorylated serines 861 and 864 as an early biomarker for neurogenesis.

In differentiating neural progenitor cells, targeting REST for degradation leads to the removal of REST from the chromatin, allowing for the expression of neuronal genes.[19] In non-neuronal cell types, REST protein is more stable - likely a critical factor contributing to its retention time on the chromatin.[9] We were interested in identifying how REST stability is maintained. A balance between kinase and phosphatase activities often regulates protein stability. Because we found that kinase activity at serines 861 and 864 to mediate REST degradation, we hypothesized that phosphatase activity may protect REST from degradation. Consistent with this hypothesis, REST has been detected colocalized on neuronal gene chromatin with the protein phosphatase CTDSP1.[20] Additionally, it is known that the expression of CTDSP1 decreases dramatically as neural progenitor cells differentiate into mature neurons,[20] while knockdown of CTDSP1 in a neural progenitor cell line accelerates neuronal differentiation.[21] We found that CTDSP1 dephosphorylates seines 861 and 864, resulting in stabilization of REST.

One of the unresolved questions in mammalian neurogenesis is how neural progenitors switch from a proliferation to differentiation state. Relevant to this question is the observation that Wnt/β-catenin signaling has different effects on neural progenitor cells depending on when it is expressed during development.[22,23] In the expansion phase of early neural progenitors, Wnt/β-catenin signaling promotes proliferation.[24-26] In the neurogenic phase, Wnt/β-catenin induces neuronal differentiation.[27]

Our results offer a model to resolve this paradox. In the expansion phase, REST is protected from degradation by CTDSP1, and remains bound to the chromatin to repress the expression of neuronal genes that would have otherwise been induced by Wnt/β-catenin signaling.[7,28,29] In subsequent neurogenic phases, an increase in ERK and Pin1 signaling levels[30] promotes βTrCP degradation of REST.[14] This results in the removal of REST from the chromatin and the derepression of neuronal genes induced by late Wnt/β-catenin signaling.[7,28,29] Future investigation of this model will provide additional resolution of the early and late stage mechanics of coordinated neurogenesis.

Implications for Advancing Regenerative Medicine in the Central Nervous System

Neural stem cells in the adult brain have the ability to generate and integrate new neurons.[31] The rate at which neurons are produced can be regulated by many factors. Exercise and mental simulation are correlated with increased neurogenesis;[32] while advanced age, stress, and diseases associated with cognitive impairment are correlated with an arrest or decrease in neurogenesis.[33] Given the flexibility in the rate at which stem cells

can generate new neurons, researchers have attempted to harness this potential to repair brain damage.[34-36] Current therapeutic approaches have identified strategies to generate new neurons in uninjured brains, including infusing or transplanting exogenous stem cells,[36] coaxing endogenous stem cells to become neurons by using blood transfusion, and supplementing growth/neurotrophic factor.[34,35,37] Unfortunately, none of these approaches have been successful in producing new integrated neurons after traumatic brain injury in humans.

Brain injury induces NPCs to generate astrocytes preferentially over neurons,[38] and these astrocytes migrate to the site of injury forming an astrocytic scar.[39] The scar protects the brain from further injury.[39] However, it also prevents neuronal regeneration at the site of injury.[40] The development of strategies directing NPC fate is an appealing approach to enhance neuronal regeneration after brain injury.

Astrocytes must maintain repression of neuronal genes. Accordingly, during astrogenesis REST is not degraded. This suggests that brain injury induces signaling cascades that promote the repression of neuronal genes. Consistent with this hypothesis, studies have found that conditions that damage the brain such as a stroke or seizure can result in derepression of REST.[41-44] The predicted outcome of this effect is the inhibition of neuronal differentiation. In fact, brain injury induces NPCs to generate astrocytes instead of neurons. An attractive and yet untested approach to promoting neuronal regeneration in brain injury is to remove this neuronal gene repression (e.g. inhibit REST).

There are many ways that inhibiting REST activity would improve neuronal regeneration therapy after CNS injury. First, it would promote the differentiation of neural progenitor cells toward neurons instead of astrocytes.[45] Second, inhibiting REST would likely reduce the risk of brain cancer associated with transplanted stem cells, according to a leading hypothesis that cancer arises from such neural stem cells.[46] In fact, insuring against tumorogenesis by transplanted cells has been a major hindrance for the adoption of brain injury-targeted stem cell therapies (FDA, http://www.fda.gov/NewsEvents/PublicHealthFocus/ucm286218.htm). Relevant to this discussion of REST regulation is the fact that many brain cancers, including glioblastoma,[47] meduloblastoma,[48,49] and neuroblastoma[50] have overexpressed REST which is implicated in oncogenic transformation.[47-49] Thus, inhibiting REST activity or expression would promote the differentiation of stem cells into neurons, reducing the risk of them transforming into tumorigenic cells. Third, there is evidence that derepression of REST in neurons promotes their death.[43] This observation indicates that inhibiting/degrading REST could protect neurons from death,[12,43] improving CNS recovery after injury.

Our Findings Establish REST As a Rational Therapeutic Target

Serines 861 and 864 determine whether REST is targeted for degradation, a critical step in neurogenesis. In the phosphorylated state serines 861 and 864 are a predicted biomarker for neurogenesis, and may help to determine the potential of neural stem cells to differentiate into neurons after brain injury. In situations where neurogenesis is repressed, it could then be reversed. In our model, we identify 2 opposing signals that regulate REST activity/stability. First, CTDSP1 protects REST from degradation by dephosphorylating serines 861 and 864 (Fig., left panel). Inhibiting CTDSP1 should therefore promote REST degradation. Second, EGF-Ras-ERK signaling phosphorylates REST at serines 861 and 864 (Fig., right panel). Thus, augmenting EGF signaling should offer a synergistic effect on neuronal regeneration.

In our study we, demonstrate the feasibility of using a peptidomimetic (decoy)

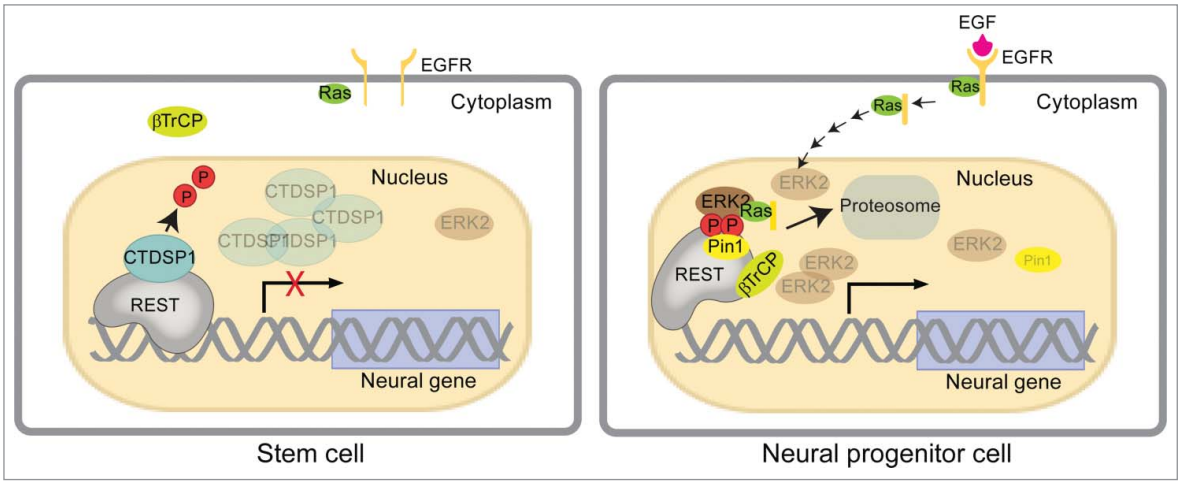

Figure 1. A model for reciprocal regulation of REST through post-translational modifications on serines 861 and 864. In stem cells, REST sits on the chromatin and represses neuronal gene expression (left panel). Here, REST is protected from degradation, because CTDSP1 dephosphorylates serines 861 and 864 on REST. During neuronal differentiation, growth factor signaling (e.g., EGF) increases activation of H-Ras and ERK, resulting in phosphorylation of serines 861 and 864. Pin1 binds to phosphorylated REST, and recruits βTrCP binding as well. βTrCP binding leads to REST degradation, allowing for expression of neuronal genes (right panel).

containing the ERK and Pin1 sites to stabilizes REST and inhibit neurogenesis. It then follows that a phosphomimetic (decoy) version of this peptide could be used to block CTDSP1 activity on REST to promote its degradation resulting in neurogenesis. As proof-of-concept, we have shown that CTDSP1 recognizes S861/864E (phosphomimetic REST). Alternatively, small molecule inhibitors of CTDSP1 activity on REST could be developed.

In sum, the mechanisms and reagents we have discovered for REST regulation reveal new elements regulating cell differentiation and provide us with new tools to influence the process of neurogenesis. Using this exciting new strategy, it may be possible to enhance neuronal regeneration after injury and interrupt the events leading to oncogenic transformation, - 2 processes that are in dire need of novel therapeutic strategies.

Disclosure of Potential Conflicts of Interest

The author is affiliated with Alcamena Stem Cell Therapeutics, LLC.

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