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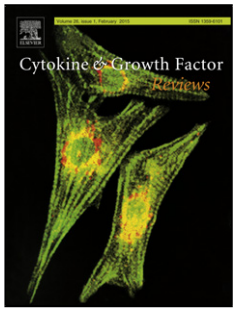


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Highlights

- HES1 is regulated by the NOTCH signalling pathway as well as the wnt and hedgehog pathways
- HES1 plays an important role in T cell development and cancer.
- HES1 represents a potent therapeutic target in cancer and leukaemia.

# HES1 in immunity and cancer

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## Abstract

Hairy and enhancer of split homolog-1 (HES1) is a part of an extensive family of basic helix-loop-helix (bHLH) proteins and plays a crucial role in the control and regulation of cell cycle, proliferation, cell differentiation, survival and apoptosis in neuronal, endocrine, T-lymphocyte progenitors as well as various cancers. HES1 is a transcription factor which is regulated by the NOTCH, Hedgehog and Wnt signalling pathways. Aberrant expression of these pathways is a common feature of cancerous cells. There appears to be a fine and complicated crosstalk at the molecular level between the various signalling pathways and HES1, which contributes to its effects on the immune response and cancers such as leukaemia. Several mechanisms have been proposed, including an enhanced invasiveness and metastasis by inducing epithelial mesenchymal transition (EMT), in addition to its strict requirement for tumour cell survival. In this review, we summarize the current biology and molecular mechanisms as well as its use as a clinical target in cancer therapeutics.

## Introduction

The basic helix-loop-helix (bHLH) proteins are comprised of several proteins that function as transcription factors and play a regulatory role in several biochemical and physiological processes- differentiation, proliferation/cell-cycle arrest and survival/apoptosis. It was studies on neuronal cells that led to the cloning of two Hairy and Enhancer of Split (HES) proteins in 1992 [1]. There are presently seven Hes proteins (HES1-7) and they all exhibit homology at the amino-acid level of the bHLH domain [1-7]. The Hes superfamily has a common proline residue within its basic motif, and the highly conserved tetrapetide domain: Trp-Arg-Pro-Trp (WRPW) at the C-terminus. Additionally, there is an 'Orange Domain' which is comprised of approximately 35 amino acids and this motif is located proximal to the C-terminus of the bHLH domain. The Orange domain thus provides an interface for protein-protein interaction [8]. HES1 is comprised of a basic DNA binding domain, followed by the helix-loop-helix (HLH) domain for dimerization (homo/hetero), the 'Orange' domain, a proline rich region and the WRPW domain.

The bHLH domain of HES1 can form homo or hetero dimers with other members of the bHLH superfamily. HES1 binds to an N-box sequence (CACNAG) or a similar N-box sequence CACGCG (denoted as the class C site) present on the promoters of genes to regulate their expression. Widely recognised as a transcriptional repressor, HES1 mediates its actions by a feedback negative loop, regulating its own expression through a tetrameric N-box site located within its promoter, to which it binds (Figure 1). Another suggested mechanism of transcriptional repression, is through the WRPW motif present at the C-terminus [9]. Here, the transducin-like enhancer of split (TLE), which is a corepressor, interacts with the WRPW motif of HES1 and leads to the recruitment of histone deacetylases (HDAC) onto the promoters of the HES1 bound target genes. This results in repression of the target genes [10, 11].

Although well known as a transcriptional repressor of the NOTCH signalling pathway, HES1 also functions as a transcriptional activator of some genes/targets and its dual role may be dependent on the interacting proteins that form the HES1 transcriptional complex. Its

activating potential has been demonstrated upon stimulation with the epidermal growth factor (EGF) where HES1 upon binding to STAT3, leads to an enhancement in gene expression [12]. Interaction between HES1 and Runt-related transcription factor 2 (Runx2) leads to an enhancement in the transcriptional activity of Runx2, inspite of HES1 being a repressor. HES1 also induces activation of PARP1 in B-cell acute lymphoblastic leukaemia (ALL) to induce apoptosis and tumor suppressive activity [13]. Thus, HES1 may function as either repressors or activators depending on the transcriptional complexes and thereby establishing a potential mechanism of cell specific dependent regulation.


Figure 1: Schematic diagram of the HES1 protein.

**Regulation of HES1**

HES1 is activated by both canonical and non-canonical pathways and NOTCH represents one of the prominent canonical pathways. Signalling mediated by the NOTCH pathway plays an important role in cellular functions such as proliferation, differentiation, survival and apoptosis [14]. NOTCH plays complex opposing roles depending on the cellular context: in mammary adenocarcinoma and T-cell acute lymphoblastic leukaemia (ALL) it functions as an oncogene whereas, in contrast, it acts as a tumour suppressor in B cell and neuroendocrine malignancies ([15-18]. Upon binding of the ligand (Jagged 1, 2, and Delta-like (Dll) 1, 3, 4) to the NOTCH receptor, there is a cascade of events which results in the exposure of cleaving sites for ADAM 10 and Y secretase resulting in the release of the intracellular domain of the NOTCH receptor (NICD). The NICD, then translocates within the nucleus and binds to transcription factors, primarily RBPjk, which leads to recruitment of co-activators/co-repressors that results in the transcription of target genes, including HES1.

The hedgehog pathway represents another mode of HES1 induction [19]. It was global gene expression studies using microarray in multipotent mesodermal cells that demonstrated the

regulation of HES1 by overexpressing sonic hedgehog [19]. This was validated using the NOTCH pathway inhibitor DAPT and chromatin immunoprecipitation studies on Gli1 targets [20]. The hedgehog pathway is activated when the morphogen hedgehog binds to the patched receptor. This results in the catalytic activity of the transmembrane protein Smoothened and subsequent activation of transcription factors, including Gli (Figure 2).

Thus, apart from the well-characterised, NOTCH and Sonic hedgehog pathways, HES1 can also be regulated by the c-Jun N-terminal kinase (JNK) signalling pathway in confluent growth arrested endothelial cells (EC) [21]. A previous study by Kim et al in 2005, had reported a link between the JNK and NOTCH signalling pathways and suggested that activation of NOTCH led to an inhibition of JNK signalling. In their study, they observed direct binding of NICD to JNK interacting protein-1 (JIP1), which resulted in inhibition of JNK [22]. The study of JNK activation in confluent EC cells, resulting in the upregulation of HES1 in a NOTCH independent manner validates the observation by Kim et al. Since NOTCH expression is suppressed in confluent EC, resulting in non existent NICD which subsequently results in the availability of JIP-1 to facilitate JNK activation.

Furthermore Stockhausen et al (2005) demonstrated that HES1 was induced by transforming growth factor-α (TGF-alpha) in a neuroblastoma cell line SKNOBE(2)c [23]. They showed that induction of HES1 in this model was dependent on the MAP kinase ERK pathway. Thus, several pathways signal to control the expression of *HES1*. There appears to be a fine cross-talk at the molecular level which is context and cell dependent, resulting in the regulation and expression of HES1.

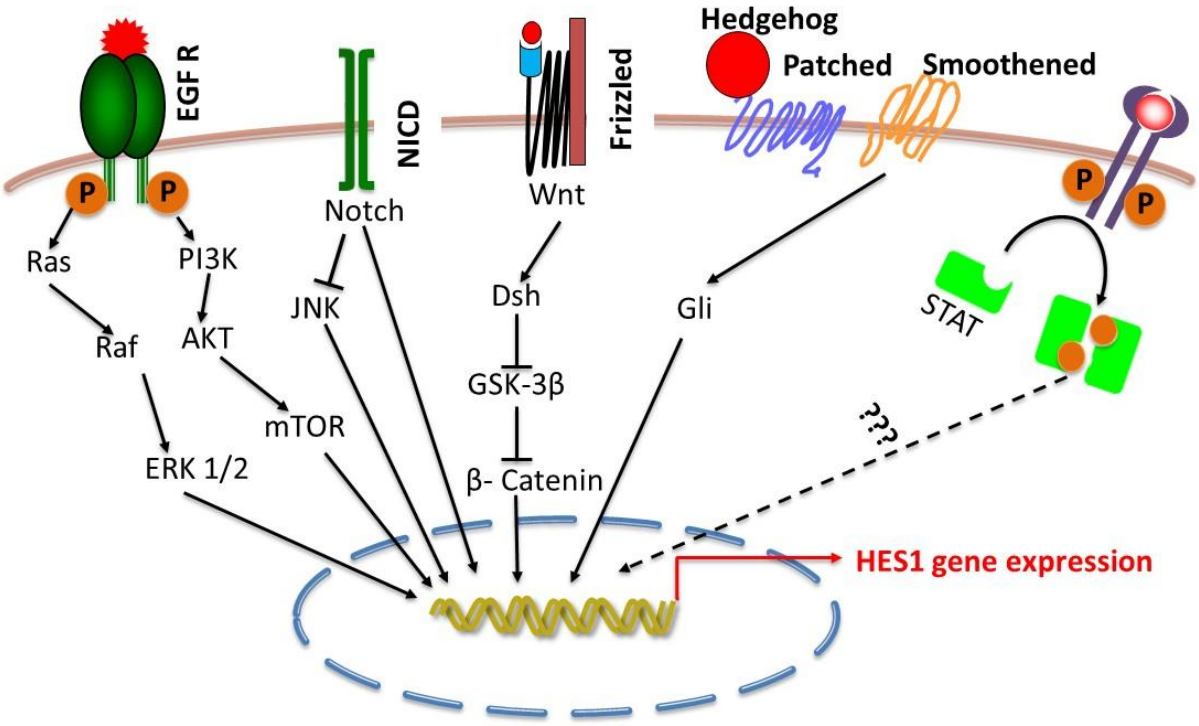


Figure 2: Regulation of HES1 at the molecular level.

**Role in immunity**

A crucial target of the NOTCH signalling pathway, HES1, is expressed in hematopoietic cells and thymic stroma and plays an important role in the development of T cells. This was confirmed in *HES1* knockout mice, where 90% of *HES1* deficient embryos presented with no thymus and <10% presented with an undeveloped thymus and here T cell development was blocked at the double negative stage (TCRβ- and TCRγδ-) [24]. A previous study in Rag1 deficient mice had demonstrated that fetal liver cells deficient in HES1 had a defective reconstitution of T cells and a culture of the fetal thymus showed that there was a decrease in the cellularity in the lobes deficient in HES1 [25]. Again, a deletion of HES1 in the bone marrow progenitor cells, resulted in a reduction (80%) in thymic cellularity [26]. HES1 deficient progenitor cells led to thymocyte differentiation being arrested at the early stages of clonal diversity, leading to a reduction in thymocyte numbers. Interestingly, NOTCH deficient mice, showed similar effects on thymocyte differentiation, leading to the notion that early stage hematopoietic cell differentiation was dependent on NOTCH mediated regulation of HES1 [27]. Kunisato et al, using alternative models reported that hematopoietic stem cell expansion could be maintained by upregulating *HES1* [28]. One of the possible mechanisms by which HES1 promotes hematopoietic stem cell differentiation, is by suppressing Cdkn1b,

which encodes the cell-cycle inhibitor $p27^{Kip1}$ [29]. A recent study by Wong et al confirmed the role of NOTCH induced HES1 and its regulation of PTEN (phosphatase and tensin homolog), a PI3K/Akt inhibitor, during normal T cell development [30].
HES1 supports differentiation of αβ T cells into CD8+ T cells, by selectively suppressing the expression of the CD4 receptor [31]. Shibata et al, identified a unique role for HES1 in T cell development and showed that HES1 plays an important role in the development of IL-17 producing γδ T cells in the thymus. It was observed that in HES1 deficient mice, the number of γδ T cells in the fetal thymus was slightly decreased and furthermore, inactivating the HES1 gene in the peripheral γδ T cells led to a reduction in IL-17 production [32].

Additionally, HES1 inhibits myeloid lineage differentiation by directly binding to and inhibiting C/EBP-α, a critical regulator of the development of myeloid and dendritic lineage cells [33]. Upon entry of progenitor cells into the thymus and subsequent signalling via the NOTCH pathway, differentiation into the myeloid lineage must be suppressed to allow for differentiation into the T cell lineages. Indeed, expressing C/EBP-α ectopically in the double-negative (DN) thymocytes via the NOTCH pathway, results in apoptosis and failure in T cell development [34]. Thus, HES1 serves as a critical prerequisite for the commitment of hematopoietic cells into various lineages.

**Role in Cancer**

HES1 is one of the most studied targets of the NOTCH signalling pathway and its upregulation has been associated with the development of several cancers including breast cancer, lung cancers, rhabdomyosarcoma, meningiomas, ovarian cancer, medulloblastoma, cervical cancer, oral squamous cell carcinoma, head and neck, colon cancer, renal cancer, pancreatic cancer, prostate cancer and cutaneous T cell lymphoma [35-39]. One of the most important transcription factors involved in NOTCH induced T cell transformation, T-ALL is HES1. Thus, aberrant activation of the NOTCH-HES1 axis results in malignancy and maintenance of cancerous cells. Interestingly, the Hedgehog-HES1 axis has also been associated with the initiation of tumours and malignancy. Most importantly, recent reports suggest a critical role of HES1 in protecting cancer cells by blocking cell differentiation inducing pathways and signals, thereby supporting the proliferation of cancerous cells [40].

It was Mackillop in 1983 who suggested that most tumours are composed of a small population of cancer stem cells, which are resistant to apoptosis, are self-regenerating and have tumorigenic properties. Several cell surface markers have been identified to be associated with cancer stem cells, CD44, CD49, CD24 and CD133 are some of them and CD144 has been associated with HES1. Indeed, a number of studies provide evidence of a pivotal role for HES1 in the progression of cancer through the induction of cancer stem cells. In colon cancer, HES1 was found to be upregulated in poorly differentiated cancer samples when compared to well-differentiated tumour samples. Again, the expression level of *HES1* positively correlated with the level of CD133 in colon cancer samples and an increase in *HES1* expression lead to a concomitant increase in the number of CD133 positive cells [36]. A previous study using lentiviral delivery of human CD133 into the rat C6 glioma cells (hCD133-C6) to produce genetically modified cell lines of rat glioma, were used to study the role of CD133 in tumorigenicity. Here, NOTCH activation and an upregulation of HES1 was observed in C6 cells that were stably expressing hCD133. Downregulating *HES1* expression, using shRNA mediated knockdown, led to a reduction in the colony forming ability of the hCD133-C6 cells. It was inferred from these studies that stably expressing hCD133 via activation of NOTCH, resulted in cell proliferation of C6 cells. This was reflected in-vivo, where tumour formation and progression was pronounced in the hCD133-C6 cells when compared to the untransfected C6 cells [41]. The importance of the NOTCH-HES1 axis was also studied in pancreatic cancer with similar observations [42]. An association of the NOTCH/HES1 role has also been reported in breast cancer and that there is an upregulation of the NOTCH receptors in breast cancer cells when compared to normal epithelia in the breast [43]. Cells expressing high CD44 but negatively stained for CD24 ($CD44^{+}/CD24^{-/low}$ cells), were resistant to chemotherapy and were most commonly found in basal-like breast tumours and had a high frequency of early relapse [44]. Moreover, activation of the NOTCH pathway is essential in the maintenance of $CD44^{+}/CD24^{-/low}$ cancer stem cells in breast cancer [45]. A recent study showed that addition of BXL0124 (Gemin Vitamin D analog) led to a reduction in the $CD44^{+}/CD24^{-/low}$ tumour initiating cells and it was confirmed that the NOTCH-HES1 axis was inhibited by BXL0124 and a subsequent reduction in the $CD44^{+}/CD24^{-/low}$ cells in basal-like breast cancer.

HES1 is involved in the tumorigenicity of stem cells in colon cancer [36]. HES1 is upregulated in metastatic prostate cancer cells, PC3 and PC3M. With the crucial role played by HES1 in tumour invasion and metastasis, it is imperative to study its role in epithelial-mesenchymal transition (EMT), since EMT is considered to be the mechanism that promotes

invasion and metastasis. Phosphatase and tensin homolog (PTEN) plays a crucial role in the regulation of EMT during embryogenesis and progression of cancer by downregulating the PI3K/AKT pathway [46]. Increasing evidence supports that inactivation or downregulation of the tumor suppressor PTEN triggers EMT in cancer, thereby promoting invasion and metastasis [47]. A recent study has demonstrated that HES1 promotes EMT related alterations by activating the AKT/PTEN axis in nasopharyngeal carcinoma (NPC) [48]. Again, HES1 has also been implicated in promoting cancer cell transformation and resistance to therapy (chemo/endocrine). One of the possible mechanisms of resistance to chemotherapy is through an upregulation of HES1, accompanied by an increase in STAT3 phosphorylation and activity [12].

HES1 is understood to promote cell proliferation by regulating factors involved in the cell cycle for example p21, p27 and CDK inhibitors of G1-S phase [29]. HES1 maintains cell quiescence by inhibiting p21 and thus preventing senescence [49]. Further mechanism is by repressing the CDK inhibitor, *CDKN1C/P57*, involved in cell cycle arrest at the G1 phase, thereby hindering p57 mediated senescence [50]. Thus, HES1 promotes and maintains malignancy through several mechanisms with a central role played by the NOTCH-HES1 axis.

**Cytokines in the regulation of HES1**

Cytokines play a crucial role in the regulation of immunity and cancer. One of the vital signalling pathway involved in these processes is the NOTCH pathway, which, in concert with several other pathways, influences the regulation of HES1. Studies have shown an upregulation of NOTCH receptors and its target genes upon stimulation with cytokines in nucleus pulposus (NP) cells [51]. Another study highlighted the dependence of NOTCH on IFN-B in Dengue viral infection. Here, they detected an upregulation of NOTCH receptors, its ligands and the target genes in infected antigen presenting cells and that the NOTCH ligands, Dll1 and Dll4 played a role in the activation and differentiation of T-cells [52]. A role for the activation of the NOTCH –HES1 pathway in cancer was observed upon the stimulation of colorectal cancer stem cells with prolactin [53]. This was found to activate the JAK2- STAT3 and ERK1/2 pathways, and cause upregulation of the NOTCH ligand, Jagged 1 and HES1. Another coactivator of interest is Maml1 which activates the NOTCH-HES1 axis. Maml1 is also known to regulate the NK-kB pathway. A recent study demonstrated that knockdown of Maml1 in the melanoma cell line M537, led to an upregulation of IFN beta

and greater migration of NK and CD8+ T cells [54]. Thus, various cytokines and transcription factors play an integrated role via the NOTCH-HES1 and target in these cytokines and pathways could serve as anticancer therapies by promoting cell differentiation and inhibiting cell proliferation

## Conclusion

The NOTCH-HES1 axis plays a crucial role in the development, differentiation and proliferation of cells along with having a vital role in stemness, metastasis and endo/chemo resistance of tumour/cancer cells. The axis represents a complex cascade of events with activators and repressors playing an important role in the comprehensive crosstalk with and between pathways. Several mechanisms that influence HES1 regulation and its effects have been studied to an appreciable extent, although many still remain at large. There could be a possible role for the signal transducers and activators of transcription (STATs). HES1 promotes and maintains the developmental proliferation of progenitor thymic T cells and various other cells, by constitutively repressing or activating transcription of cell cycle regulators. With its critical role in cancer cells, it serves as a biomarker and a very potent target for cancer therapy.

# Biographies

## Aradhana Rani – Short Biography

After a PhD at King's College London in 2010, Aradhana continued at King's as a Research Fellow in Dr Stipo Jurcevic's lab where she worked on clinical trials and also continued her research in cytokine biology. In 2012, she was awarded a Postdoctoral Investigator award by the Cytokine Society in Geneva, Switzerland.

To gain experience in cancer biology, she joined the Institute of cancer Research in the extended lab of Professor Mitch Dowsett for a period of a year in 2014. She subsequently joined the University of Westminster as a Visiting Lecturer. She is part of the research group of Dr Richard Smith, Dr Christine Galustian and Professor Prokar Dasgupta within the MRC Centre for Transplantation and is also involved in teaching.

Christine Galustian – Short Biography

After a PhD at Imperial College, Christine started at Northwick Park hospital at the MRC Glycosciences laboratory working with Ten Feizi as a non-clinical MRC fellow – and helped in the development of the first Glycomic chips.

After 8 years at Northwick Park, Christine became team leader of a group funded by Celgene at St Georges in London, working with Angus Dalgleish on the development and mechanisms of action of immunomodulatory drugs, the most famous being Revlimid, now FDA approved for multiple myeloma. Still have an honorary academic appointment there and continue to collaborate with Professor Dalgleish to develop novel combinatory immunotherapeutic regimens

Christine is now working at Kings College London as a PI and team leader along with Dr Richard Smith and Professor Prokar Dasgupta within the MRC Centre for Transplantation to develop novel non-toxic immunotherapies for Prostate Cancer. The group have generous funding from the Prostate Cancer research centre charity, Prostate Cancer UK, the MRC and the NIHR.

Richard Smith gained his DPhil. from Oxford University in photoaffinity labelling and antibody structure. He has spent most of his career in the pharmaceutical and biotechnology industries and he played a major part in the discovery and development of the thrombolytic agent Anistreplase and the complement inhibitor Mirococept. The latter is an application of cytotopic protein modification technology which he has pioneered. After a long-standing collaboration with the Department of Nephrology and Transplantation, he joined the Department in 2007 to set up the new Protein Therapeutics Laboratory of which he is the director.

Roseanna Greenlaw is a post-doctoral research associate at King's College London (KCL). She did her PhD with Sir Professor Robert Lechler in transplant immunology at the Royal Postgraduate Medical School, London followed by a post-doctoral position in the Dept of Immunity and Infection with Professor Maggie Dallman at Imperial College London. She then joined KCL and worked as a non-clinical research fellow for Dr Stipo Jurcevic in the Dept of Nephrology and Transplantation with an interest in developing novel antibody combination therapeutics for the pre-sensitized transplant recipient.
She has always been most interested in translational research and has been involved in clinical trials since 2001. In February 2013, she joined Dr Richard Smith's Protein Therapeutics lab and is currently a member of the EMPIRIKAL trial research team, investigating the efficacy of Mirococept for preventing ischemia-reperfusion injury in the kidney allograft.

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