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Relevance of HEY Proteins for Notch4 mediated regulation of Slug and Twist1 in melanoma

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Abstract

Melanoma is the most aggressive form of skin cancer and information to the pathogenesis of advanced disease is still sparse. During progression, melanoma cells may undergo a epithelial-mesenchymal-like transition (EMT) in order to gain enhanced migratory capacity, invasiveness and increased resistance to apoptosis. These abilities are necessary for tumor cells to disseminate and form metastases. At the stage of extravasation a phenotypic switch via mesenchymal-epithelial-like transition (MET) might be necessary to reverse into cells with higher proliferative capacity. One of the signaling pathways implicated in EMT is the highly conserved Notch pathway which plays an important role in development and tissue homeostasis under normal physiological conditions. Preliminary data show that the epithelial mesenchymal transition regulators (EMTR) Slug and Twist1 are regulated through the Notch signaling pathway. Further, Notch4 leads to up regulation of Hey1 and Hey2, two transcription factors, which have been shown to be regulated by Notch.

The specific aim of this project was to investigate the Notch4 mediated regulation of Slug and Twist1 in melanoma cell lines in more detail. To this purpose the importance and involvement of Hey1 and Hey2 in the regulation of Slug and Twist1 was investigated by silencing and overexpression experiments. The effects of these experiments were then assessed on protein and mRNA levels using immunoblotting and quantitative real-time reverse transcription polymerase chain reaction (real-time RT-PCR), respectively. Additionally the interactions of Hey1 and Hey2 proteins with the promoter regions of Slug and Twist1 were examined using electrophoretic mobility shift assays (EMSA). Finally the effect of the protein DNA interactions on the promoter activity of Slug and Twist1 were assessed by performing luciferase reporter gene assays.

The experiments showed that both Hey proteins have suppressive effects on the investigated EMTRs. These effects were in line with the observations of the respective Notch4 experiments which suggest that Notch4 negatively regulates Slug and Twist1 by regulating Hey1 and Hey2. This hypothesis is strengthened by the results of EMSA which show that the Hey proteins indeed interact with the promoter regions of Slug and Twist1. Additionally decreased activity of the Slug and Twist1 promoters in Hey overexpressing cells was found in luciferase assays, while mutation of the respective E-boxes partly restored the activity.

Taken together the acquired results suggest that Notch4 is suppressing Slug and Twist1 in indirectly through up regulation of Hey1 and Hey2. The suppression of EMTRs like Slug and Twist1 shows that Notch4 is involved in the regulation of the mesenchymal-epithelial-like transition. For the first time, these results suggest an important role of Notch4 as a tumor suppressor in melanoma highlighting the importance and diversity of Notch signaling in melanoma, shedding new light on the proposed deployment of Notch inhibitors for melanoma therapy.

Zusammenfassung

Die genaue pathologische Entwicklung von Melanomen, der tödlichsten und aggressivsten Hautkrebsart, ist noch immer unzureichend erforscht. Nach heutigem Wissensstand müssen Melanomzellen im Rahmen der Metastasierung einen Prozess durchlaufen, welcher der sogenannte Epithelialen-Mesenchymalen Transition (EMT) ähnlich ist. Dabei wechseln die Zellen von ihrem ursprünglich epithelialen Phänotyp in einen mobileren und invasiveren mesenchymalen Phänotyp. Diese mesenchymalen Melanomzellen breiten sich über die Blutbahn aus und siedeln sich in weiterer Folge in entfernten Organen und Geweben an. In den Organen wechseln Melanomzellen im Zuge der Metastasenbildung danach durch eine Umkehrung des EMT Prozesses in ihren ursprünglichen Phänotyp zurück, wobei man dabei von der Mesenchymalen-Epithelialen Transition (MET) spricht. Unter physiologischen Bedingungen wird der EMT Prozess, welcher auch bei der Wundheilung und in der embryonalen Entwicklung wichtig ist, unter anderem durch den Notch Signalweg gesteuert.

Erste Untersuchungen haben gezeigt, dass die Transkription der EMT Regulatoren (EMTR) Slug und Twist1 vom Notch Signalweg gesteuert werden. Das Ziel dieses Projektes war es die Notch4 vermittelte Regulation von Slug und Twist1 in Melanomzellen zu eruieren. Ein besonderer Fokus des Projekt war die Beteiligung der Hey Proteine (Hey1 und Hey2) an diesem regulatorischen Prozess. Die Effekte der eventuell beteiligten Faktoren Notch4, Hey1 und Hey2 wurden durch „knockdown“ und Überexpressionsversuche auf der Protein- als auch auf der mRNA Ebene mittels Immunoblottings und quantitativer Real-Time reverse transcription Polymerase-Kettenreaktion (real-time RT-PCR) bestimmt. Zusätzlich wurden die Protein-DNA Interaktionen der Hey Proteine mit Fragmenten der Slug und Twist1 Promotoren mithilfe des electrophoretic mobility shift assays (EMSA) untersucht, wobei in weiterer Folge die Auswirkungen dieser Interaktionen auf die Promotoraktivität mit Hilfe des Luciferase reporter gene assays ermittelt wurde.

Die im Rahmen dieses Projektes durchgeführten „knockdown“ und Überexpressionsexperimente zeigten, dass sowohl Notch4 als auch die bekannten Notch induzierten Proteine Hey1 und Hey2 die EMTRs Slug und Twist1 supprimieren. Des Weiteren konnte die direkte Interaktion zwischen Sequenzen der Slug und Twist1 Promotoregionen und der Hey Proteine mittels EMSA nachgewiesen und durch Luziferase Assays bestätigt werden. Die Promotoraktivität der untersuchten EMTRs wurde durch Überexpression der Hey Transkriptionsfaktoren stark reduziert.

Zusammenfassend kann gesagt werden, dass die erhaltenen Daten zeigen, dass Slug und Twist1 durch Notch4 indirekt reguliert werden. Diese Regulation wird durch die bekannten Notch induzierten Transkriptionsfaktoren Hey1 und Hey2 vermittelt und ist gleichzeitig ein Hinweis auf eine mögliche tumorsuppressive Funktion von Notch4 in Melanomen. Diese neu gewonnenen Erkenntnisse könnten einen wichtigen Beitrag zur Entwicklung von Melanomtherapien leisten und sollten vor allem bei der Verwendung von Notchinhibitoren berücksichtigt werden.

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Rationale

Notch signaling is a highly conserved signaling pathway that plays important roles during development and tissue homeostasis but has also been reported to be involved in oncogenesis in different tumor types [1]. Considering the highly diverse effects of Notch signaling during development it is no surprise that there are also reports about tumor suppressive functions in different but also same cancer types. The highly context dependent outcome of Notch signaling ranges from differences between tissue types to differences of receptor types in the same tissue [2, 3].

In melanoma Notch1 has been reported to be a major factor involved in cancer development and progression [4]. Additionally an oncogenic role of Notch4 in melanoma has been described by regulating the embryogenic morphogen Nodal [5]. Due to the presented evidence of the oncogenic role of Notch in melanoma a phase II trials using broad Notch signaling inhibitors have already been performed but only showed minimal clinical activity against metastatic melanoma [6].

Therefore more in depth knowledge about the detailed functions of Notch signaling in melanoma are required in order to understand and increase the efficiency of melanoma treatment by targeting the Noch signaling pathway.

The presence of the conserved CSL binding sequence in both promoter regions of Slug and Twist1 suggests the involvement of the Notch signaling pathway in the regulation of these EMTRs. Since Slug and Twist1 are reported to be inhibitors of the E-cadherin expression and therefore are involved in the epithelial-mesenchymal transition [7, 8] the regulation of these EMTRs could yield valuable information about tumor progression and metastasis formation mediated by Notch.

1 Introduction

1.1 Notch signaling

Notch is one of the fundamental and evolutionary conserved signaling pathways that regulate development and adult tissue homeostasis. It is involved in a variety of important cellular mechanisms like cell fate specification, differentiation, proliferation, apoptosis, adhesion, epithelial-mesenchymal transition, migration, and angiogenesis. [1]. The outcome of the Notch signaling in individual cells is often quite different and is highly dependent on signal dose and context. The effects can range from increased survival to death, proliferation to growth arrest and from commitment to differentiation to blockage of differentiation [9]. A clear sign for the importance of the Notch pathway is the embryonic lethality of deficiencies in Notch signaling in various model organisms, including worms, flies and mice [10].

1.1.1 The Notch Signaling Pathway

Notch signaling is activated through short range cell-cell communication. Both the Notch receptors as well as the Notch ligands are modular single transmembrane proteins [10]. Interaction of the Notch receptor on the signal receiving cell with the ligand of the signal sending cell leads to proteolytic cleavage of the receptor molecule. In this process the receptor is cleaved twice. The first cleavage is carried out by a metalloprotease of the ADAM family (A Disintegrin And Metalloproteinase) and takes place at the juxtamembrane region of the extracellular domain. This cleavage detaches the extracellular domain of the Notch receptor from the signal receiving cell and induces sensitivity of the truncated receptor to a second cleavage step. This second cleavage is then carried out by the γ-secretase multiprotein complex [10]. This complex consists of presenilin, nicastrin, PEN2 and APH1 [11]. The γ-secretase cleaves the Notch receptor at an intramembrane cleavage site called S3 which releases the Notch intracellular domain (NICD) from the membrane. The NICD then translocates into the nucleus were it interacts with the core transcription factor of Notch signaling CSL (C-promoter-binding factor/Suppressor of

hairless/Lag1). Upon binding of the NICD to CSL it changes its function from a transcriptional repressor to a transcriptional activator. In order to activate the target gene CSL recruits its co-activator Mastermind (Mam) and other co-activators like p300 [10]. Figure 1 summarizes the events after ligand binding to the Notch receptor.

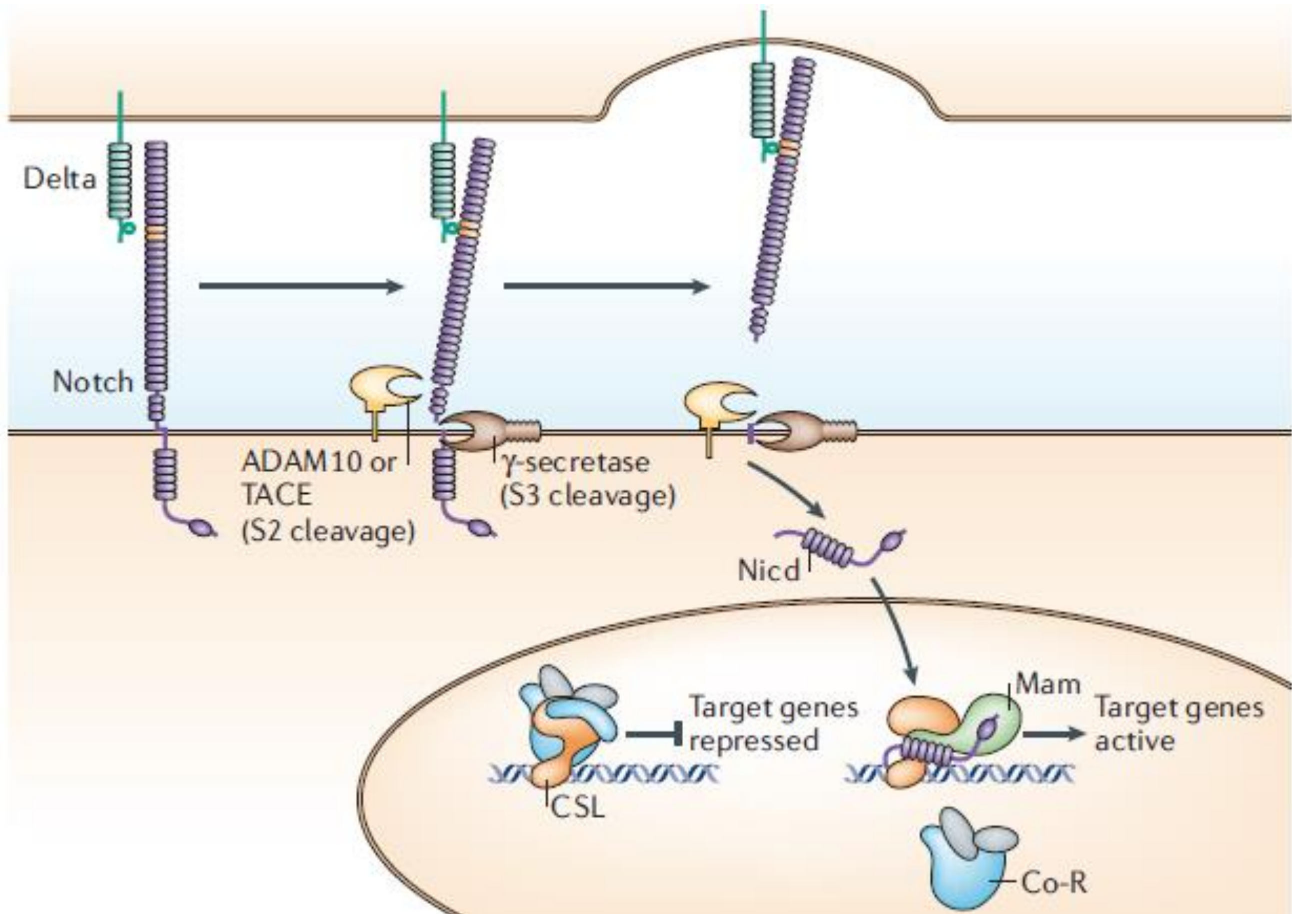


Figure 1: Summary of Notch signal transduction. *After receptor-ligand binding a metalloprotease of the ADAM family like TACE (TNF-α-converting enzyme) or ADAM10 cleaves the ligand bound receptor at the S2 cleavage side on the extracellular domain. This cleavage generates a substrate for the γ-secretase complex which cleaves the truncated receptor at the intramembrane cleavage side S3. This releases the Notch intracellular domain (NICD) from the membrane which translocates to the nucleus and interacts with CSL. This interaction triggers the exchange of co-repressors with co-activators like mastermind (Mam) and leads to target gene activation. [11]*

1.1.2 The Notch Receptor family

In mammals there are four Notch receptors while there is only one in *Drosophila* and two in *C. elegans* [12]. All these receptor Proteins are proteolytically modified in their maturation process by a furin like convertase in the trans-Golgi network. The furin like convertase cleaves the receptor protein at the S1 cleavage site which generates the mature receptors that are noncovalently linked heterodimeric proteins consisting of an Notch extracellular domain (NECD) and a Notch transmembrane and intracellular domain (NTMIC) [12, 13]. The extracellular domain of all Notch receptors includes epidermal growth factor (EGF)-like tandem repeats that are involved in ligand binding. These EGF-like domains are about 40 amino acids long and contain six cysteine residues that from characteristic disulfide bonds. The receptors in *C. elegans* (cLIN-12 and cGLP-1) both have much shorter extracellular domains than the receptors in mammals and in *Drosophila*. The length differences are due to the different numbers of EGF-like repeats present in the different species (11-14 repeats in the *C. elegans* receptors compared to 29-36 repeats in the mammalian and *Drosophila* receptors). Beside these EGF-like repeats the extracellular domain consists of the negative regulatory region (NRR) which is located between the transmembrane domain and the EGF-like repeats [10]. This region contains three Lin12/Notch repeats (LNRs) and the heterodimerization domain (HD). The HD domain harbors the S1 and S2 cleavage sites with S1 dividing the domain in two subdomains HD-N and HD-C. The NRR in general is responsible for the metalloprotease resistance in the absence of ligand binding [14]. The Notch intracellular domain (NICD) consists of multiple conserved functional regions that are shared among the different Notch receptors of different species. These regions include the RAM domain (recombination binding protein-Jκ-associated molecule) which, together with the seven ankyrin repeats (ANK), is responsible for the binding of the transcription factor CSL. The ANK repeats are flanked by nuclear localization signals (NLS).

Additionally the NICD contains a less conserved trans-activation domain (TAD) that differs between the notch receptors as well as a C-terminal PEST motif that negatively regulates protein stability [10, 15]. Even though the C-terminal regions of the Notch receptors differ in their domain structure (especially the TAD domain is missing in Notch3 and Notch4) comparative in vivo analysis have not revealed any functional differences [16]. However the TAD domain has been reported to be essential for physiological Notch1 function [17] which indicates the possibility for different activation patterns of the different Notch receptors.

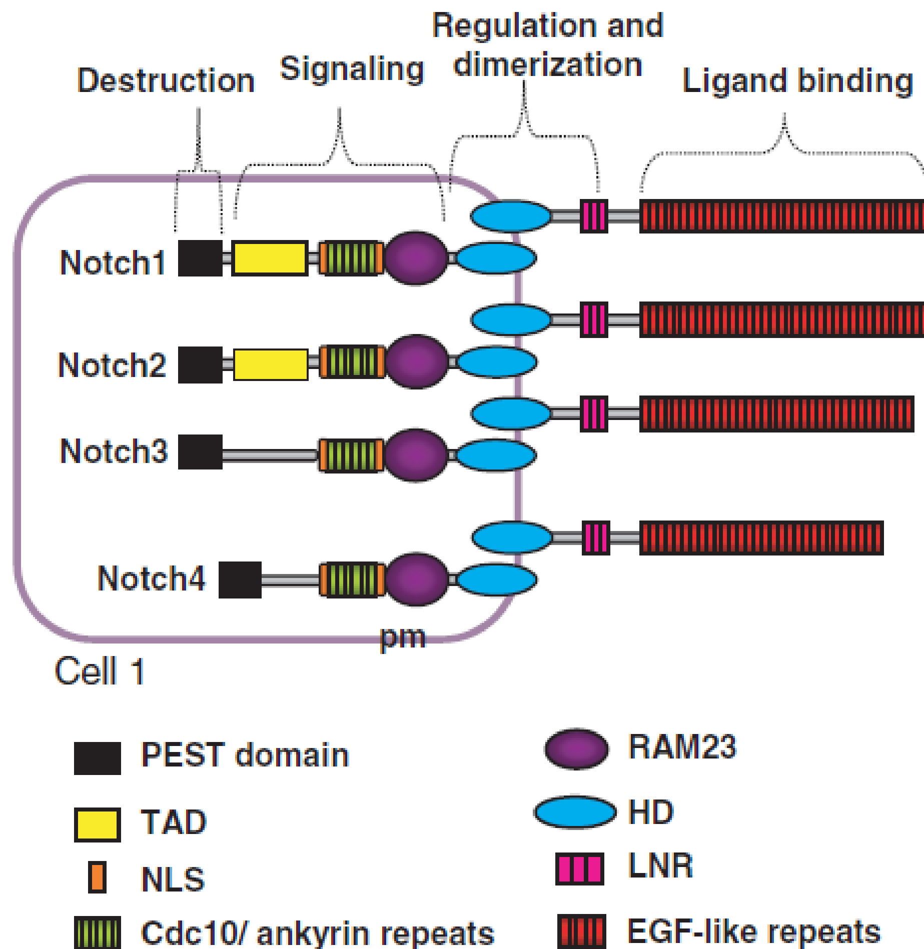


Figure 2: Schematic representation of mammalian Notch receptors. *The extracellular domain consists of a different number of EGF-like repeats (36 in Notch1 and 2; 34 in Notch 3; and 29 in Notch 4) which are responsible for ligand binding as well as the negative regulatory region (NRR) marked as regulation and dimerization region. Then intracellular domain consists of the highly conserved RAM and ankyrin segments as well as the variable TAD domain which are responsible for the signal transduction and the C-terminal PEST sequence which negatively regulates protein stability. The plasma membrane is indicated with a violet line and marked as pm. [1]*

1.1.3 The Notch ligands

In general the majority of Notch signaling is mediated through the canonical Notch ligands. Beside these canonical ligands, there is an increasing number of non-canonical ligands that also affect Notch signaling [18].

Canonical Notch ligands

The canonical Notch ligands, the so called DSL family ligands, are named after their first identified members Delta, Serrate (both *D.melongaster*) and Lag2 (*C.Elegans*). Similar to the Notch receptors, the canonical Notch ligands are type 1 transmembrane proteins that are located at the cell surface. Like Notch receptors Notch ligands have tandem EGF-like repeats on their extracellular domains but also contain two other important domains that are necessary for the ligand-receptor interaction. These domains are the MNNL (**M**odule at the **N**-terminus of **N**otch **L**igands) domain and the DSL (Delta/Serrate/Lag2) domain. These two domains as well as the first two EGF-like repeats are required for successful binding of Notch receptors.
Based on the structural homology of the mammal Notch ligands to the ligands Delta and Serrate of *D.melanogaster* they are divided in either Delta like or Serrate like ligands. Mammals possess two Serrate like ligands called Jagged 1 and Jagged 2 as well as three Delta like ligands called Dll1 Dll3 and Dll4. The main difference between these ligand types are the number of EGF like repeats in which the Serrate like ligands have almost twice as many repeats as the Delta like ligands. Additionally the Serrate like ligands have a cysteine-rich region (CR) at the C-terminal end of the extracellular domain, which has partial homology to the Von Willebrand Factor type C domain (VWFC).
The intracellular domains of the DSL ligands show almost no conserved domains. However many ligands contain multiple lysine residues and a C-terminal PDZ (PSD-95/Dlg/ZO-1) ligand motif which are responsible for signaling activity and cytoskeleton interactions, respectively [18].

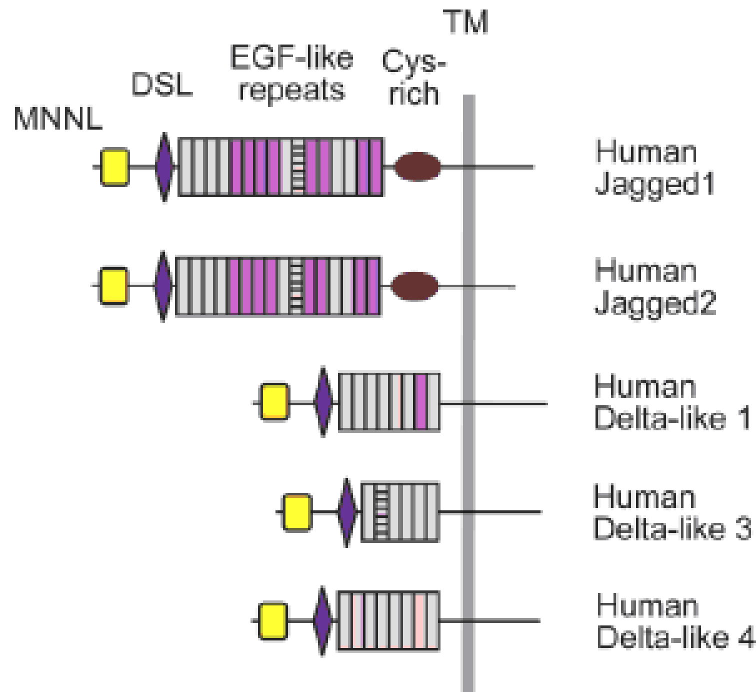


Figure 3: Schematic representation of the canonical notch ligands. *Shown are the two Serrate like ligands, Jagged 1 and Jagged 2, as well as three Delta like ligands, Dll1 Dll3 and Dll4. The extracellular domains consist of the tandem EGF-like repeats. Additionally there are the MNNL (Module at the N-terminus of Notch Ligands) domain and the DSL (Delta/Serrate/Lag2) domain that both are necessary for ligand-receptor interaction. The serrate like ligands have a Cys rich domain close to the transmembrane segment. All canonical notch ligands show almost no conserved domains on their intracellular segment. The C terminal PDZ (PSD-95/Dlg/ZO-1) ligand motif is not included in the figure. [10]*

Non-canonical Notch Ligands

Notch signaling is used very frequently in many different situations which are difficult to reconcile with the low number of canonical Notch ligands and receptors expressed in mammals. One possible explanation for the high diversity of the notch signaling pathway is the presence of non-canonical Notch ligands, which compared to the canonical ligands, are structurally much more diverse and include integral membrane, glycosylphosphatidylinositol (GPI)-linked, and even secreted proteins [18].

1.1.4 Regulation of Notch receptor activity

One of the possible regulatory mechanisms of the Notch receptor are cleavage events during the signaling process described earlier. Both, the γ-secretase complex as well as the ADAM familiy members ADAM 10 and 17 show potential for regulation through external factors, membrane environment or intracellular pathways. However the in vivo regulation of these proteases still requires extensive investigation [11].
Besides the proteolytical cleavage during the signaling event there are a number of other possible regulatory mechanisms. One of these mechanisms is glycosylation. The EGF-like repeats of the extracellular domains of the receptors are possible glycosylation sites. The Enzyme O-Fucosyl transferase (O-Fut) is essential to generate functional Notch receptors, which is highlighted by the embryonic lethality of O-Fut deficient flies and mice [19]. It adds the first fucose residue and therefore enables the successful post translational modification. Additional to the important enzyme function, O-Fut also acts as a chaperone supporting the folding and the transport of the Notch receptors from the endoplasmatic reticulum to the cell membrane. Following the addition of the first fucose residue the carbohydrate chains can be elongated by glycosyl transferases of the fringe family. The number of the possible glycosylation sides of the Notch extracellular domain can lead to a very diverse glycosylation pattern that has been shown to alter the receptor activity [11]. A Notch mutation that introduces a fucosylation site at the Notch extracellular domain in neural cells of *D. melanogaster* lead to an ectopically active receptor emphasizing the importance of the glycosylation for the Notch receptor activity [20]. Ubiquitination is another important regulatory mechanism in the Notch signaling. The E3 ubiquitin ligase Deltex or Itch/AIP4 (Atrophin-1 interacting protein 4) can for example poly-ubiquitinate non-activated Notch in the cytoplasm. This poly-ubiquitination will result in endocytosis of the non-activated Notch receptor which will ultimately result in either lysosomal degradation or recycling to the plasma membrane. Additionally the enzyme Fbw7 but also Deltex and Itch can ubiquitinate active Notch targeting it for proteasomal degradation, which leads to a rapid turnover of the active NICD. Beside the ubiquitination, phosphorylation, acetlylation and hydroxylation of the NICD have also been reported but are currently less well understood [21].

1.1.5 The DNA binding factor CSL

The transcription factor CSL is named according to the mammalian, *D. melanogaster*, and *C. elegans* orthologous proteins (CBF-1/RBP-jκ, Su(H), Lag-1). Located in the nucleus, CSL binds the conserved DNA sequence (C/A/T)(G/A)TG(G/A/T)GAA [22], where it acts both as a transcriptional repressor and activator. In the absence of the NICD, CSL is bound to its binding sequences and recruits co-repressor proteins to form a multi-protein repressor complex that presumably affects the chromatin structure by recruiting histone deacetylase complexes (HDAC) which results in transcriptional repression. Co-repressors that have been shown to interact with CSL are SMRT (silencing mediator of retinoid and thyroid receptors)/N-CoR (nuclear receptor co-repressor), CIR (CBF1-Interacting co-Repressor), and SPEN (also known as SHARP, SMRT/HDAC-1-associated protein) [23].

Upon Notch signal activation, the cleaved NICD translocates into the nucleus where it interacts with CSL and replaces the co-repressor proteins. This subsequently triggers the binding of the co-activator Mastermind (MAML) which results in the assembly of a transcriptional active ternary complex. This complex is capable of recruiting general transcription factors like CBP/p300 and therefore activates the corresponding Notch target gene (Figure 2). Additionally the transcriptional co-regulator SKIP (Ski-Interacting Protein) is assumed to be involved in both the repressor and activator complex and bridges either interactions between CSL and NICD or CSL and co-repressors [23]. The MAML protein further regulates the hyperphosphorylation of the NICD PEST domain which subsequently leads to ubiquitin ligase-mediated degradation of the NICD and halts the signaling event. Considering its essential role in the repression and activation of the canonical Notch signaling, CSL can be considered as a transcriptional switch in the regulation of Notch target genes, where it acts as the centerpiece for the protein-protein interactions that are necessary in order to modulate gene activity. [24, 25].

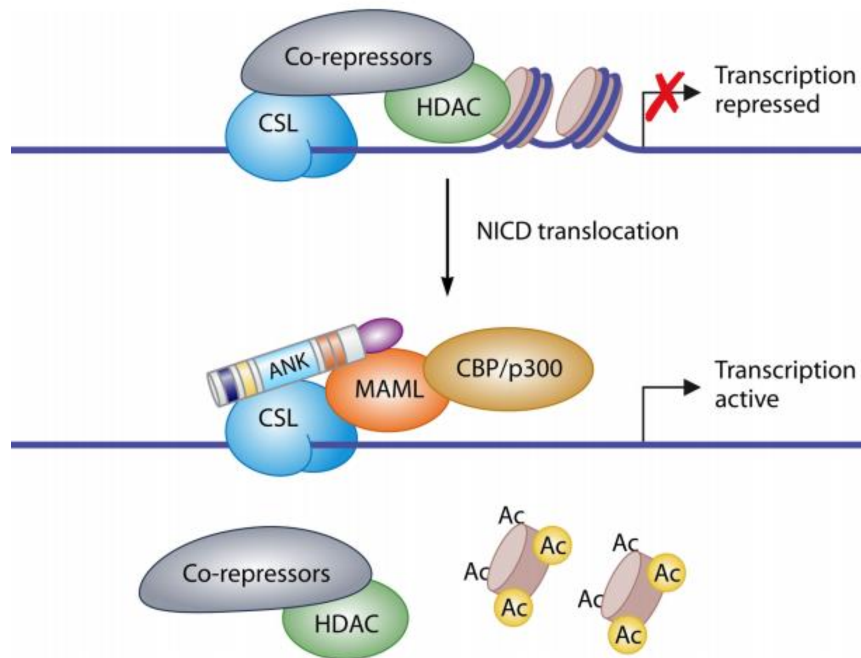


Figure 4: Schematic representation of the CSL binding complexes that modulate Notch target gene expression*. In the absence of NICD, CSL binds co-repressors which recruit histone deacetylases and therefore lead to transcriptional repression. Binding of NICD to CSL displaces the co-repressors and triggers the formation of a ternary complex with CSL and Mastermind (MAML). This complex then recruits co-activators like CBP/p300 which leads to transcriptional activation [25].*

1.1.6 Notch target genes

The Notch signaling pathway is a highly complex network that includes crosstalk to other distinctive signaling pathways like Wnt or hypoxia induced signaling. An intrinsic part of this signaling network is the feedback loop mechanism that can alter the network positively or negatively at different stages of signal transduction (signaling initiation, stability of the NICD or co-regulation of Notch target genes). Notably, part of the Notch target genes are the Notch receptors itself as well as the Notch ligands Jagged1 and DLL1. Furthermore some of the Notch target genes are transcriptional suppressors like members of the Hes and Hey family of transcription factors. These factors, especially Hes1, are involved in a phenomenon called “incoherent network logic” that describes their ability to suppress the transcription of the signal initiating factor (Notch receptors) as well as itself, creating a “window” for signal responsiveness [26].

The Hes (Hairy/Enhancer of Split) and Hey (Hairy/Enhancer-of-split related with YRPW motif) families of basic helix loop helix (bHLH) transcription factors belong to the best characterized Notch target genes. Members of both families are regulated by Notch in a CSL dependent manner which, upon Notch activation, leads to the suppression of the corresponding Hes/Hey target genes [27].

The Hey family consists of three members (Hey1, Hey2 HeyL) that all share structural key features typical for bHLH transcription factors. The DNA binding is mediated by the N-terminal basic domain which is directly followed by a helix loop helix domain that is responsible for the necessary dimerization of the transcription factors. Another highly conserved domain of the Hey family is the so called orange domain. This domain presumably is also involved in protein-protein interactions and modulates dimerization affinity. The majority of the C-terminal part of the Hey proteins shows no significant conservation. However the last few amino acids show the conserved sequence YRPW of which the function is not identified as of yet [28]. This YRPW motive (compared to the WRPW of Hes) as well as the replacement of a critical proline residue by a glycine residue in the basic domain is the main difference of Hey and Hes proteins. The change in the YRPW motive renders the Hey proteins unable to bind the TLE co-repressors. Additionally the amino acid substitution in the basic domain alters the DNA binding of the transcription factors. While Hes proteins are able to bind N- and E-boxes (CACNAG, CANNTG), Hey proteins are unable to bind N-box sequences [29]. The preferred binding sequence of the Hey proteins involves the class B E-box sequence with specific conserved flanking nucleotides that are conserved from the Drohsophila homologue E(spl) and is tggCACGTGcca [30, 31].

The biological function of Hey proteins is mainly suppression of target genes. However the mechanism behind the suppression as well as the target gene specification are highly variable. Like most bHLH transcription factors, Hey proteins form homo and heterodimers. Especially the heterodimers with Hes family members are stable and lead to different affinities for different DNA sequences resulting in a broad spectrum of possible target genes [29]. However interactions with other bHLH transcription factors have also been reported. Hey1 forms dimers with MyoD, a muscle specific factor that is active during myogenic differentiation. This interaction

prevents the formation of a critical MyoD/E47 heterodimer which results in suppression of MyoD target genes [32].

Hey2 on the other hand has been reported to interact and suppress the activity of the zinc finger transcription factors GATA4/6 in the developing heart. Hey2 knockout mice showed up regulated GATA target genes confirming the suppressive effect of Hey2 on GATA [33]. Additionally Hey and Hes proteins are able to interact with a number of repressor proteins like mSin3A and N-CoR or SIRT1 and TLE1 highlighting the diversity of the Hey mediated regulatory functions [28]. Figure 5 shows a summary of the Notch mediated indirect suppression of Hey/Notch target genes by Hey/Hes transcription factors.

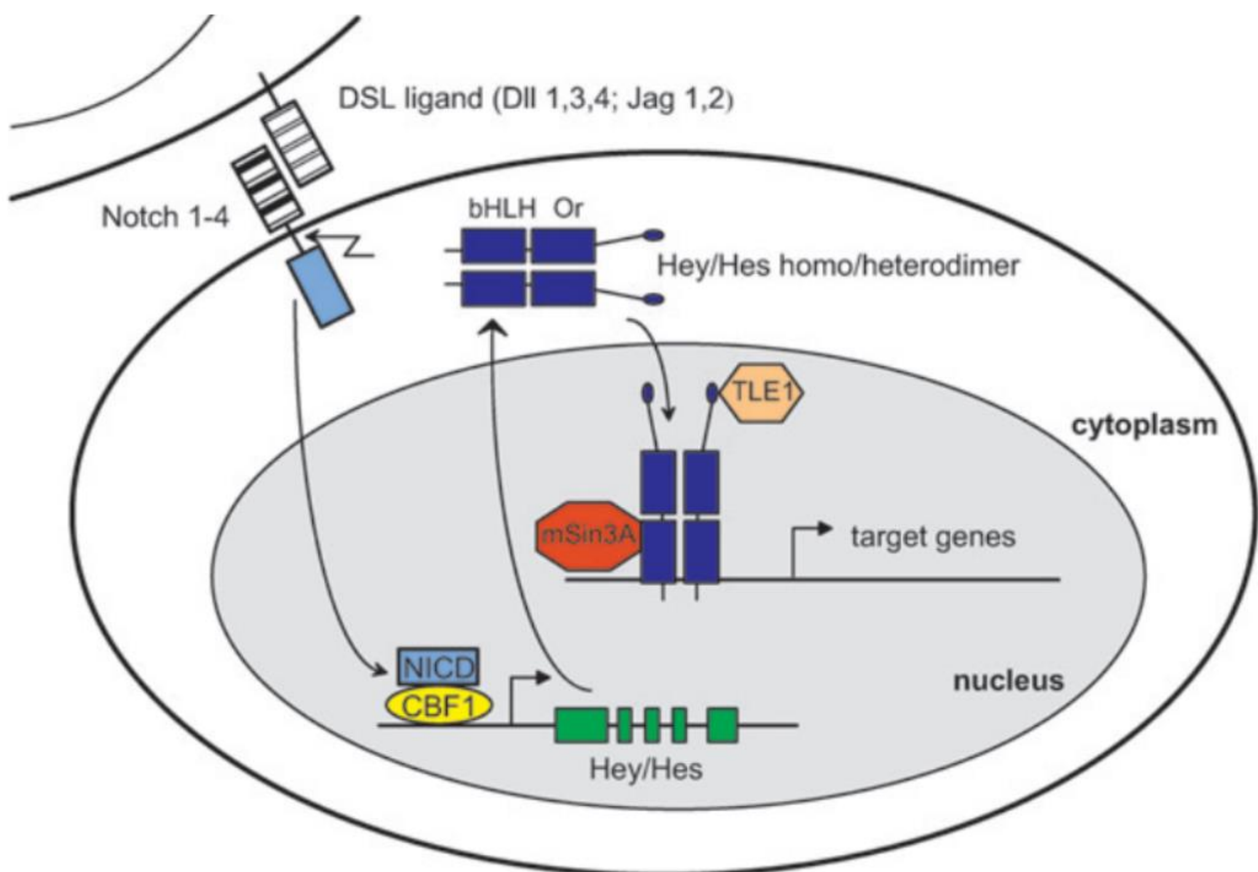


Figure 5: Notch mediated indirect suppression of Hey/Notch target genes by Hey/Hes transcription factors*. Upon activation of Notch signaling the NICD is released and translocates into the nucleus. There it interacts with the transcription factors CBF1 (CSL) and activates the transcription of the Hey/Hes bHLH transcription factors. The active Hey/Hes proteins form homo or hetero dimers and again translocate into the nucleus. After specifically binding target DNA sequences the transcription of the corresponding gene is suppressed by the recruitment of co repressors like mSin3A or TLE1. [28]*

1.1.7 Notch oncogene or tumor suppressor?

The oncogenic role of Notch has first been identified in T-cell acute lymphoblastic leukaemia (T-ALL). These cancer type harbors a specific chromosomal translocation [t(7;9)(q34;q34.3)] that leads to the expression of a truncated Notch1 receptor. This constitutively active receptor drives haematopoietic progenitor cells into the T-cell lineage which has been confirmed by loss of function experiments [2]. Furthermore T-ALL can be induced by proviral integration into the locus of Notch receptor genes. For example the integration of the feline leukemia virus into the Notch2 gene results in Notch2 transcription regulated by the viral promoter which leads to the development of the disease [34].

One of the first reports of the involvement of Notch signaling in solid tumors describes an integration of the mouse mammary tumor viruses (MMTVs) next to the "*Int-3*" locus, which resulted in a constitutively active *Notch4 receptor,* ultimately leading to breast cancer development [35]. The Notch signaling pathway is also found to be involved in the major subtype of lung cancer, the lung adenocarcinoma (LAC), development. Recent studies show an oncogenic role of Notch1 and Notch3 in the initiation and maintenance of LAC which belongs to the non-small-cell lung cancers (NSCLC) [36, 37]. Benign to malignant cutaneous melanocytic lesions show a significant increase of Notch receptors, especially Notch1 and Notch2, and the Notch ligands Jagged-1, Jagged-2 and Delta- like 1, suggesting an oncogenic role of Notch in melanoma development and progression [4]. The oncogenic role of Notch1 is presumably mainly mediated through the up regulation of beta-catenin and N-cadherin. Additionally it has been shown that Notch can positively regulate Slug, a transcriptional repressor that is known to repress E-cadherin. Increased N-cadherin and decreased E-cadherin are highly correlated with melanoma progression and metastasis formation [38]. More recent studies have also described a direct activation of neuregulin1 (NRG1) by Notch1. Inhibition of NRG1 leads to delayed tumor growth, while expression of recombinant NRG1 partly restores melanoma cell growth after Notch1 knockdown. On the molecular level these effects are reported to be at least partly mediated through the PI3Kinase/Akt pathway [39]. Moreover oncogenic functions of Notch have been described in liver, pancreatic, colorectal as well as different types of hematopoietic cancers [40].

In contrast to the previously described tumor facilitating effects of the Notch signaling pathway, recent evidence also shows tumor suppressive functions of Notch. Human acute myelogenous leukemia (AML) is one example in which Notch has a tumor suppressive effect. In AML activation of Notch receptors or the downstream Notch target gene Hes1 leads to a down regulation of B cell lymphoma 2 (BCL2) and an up regulation of p53/p21 ultimately resulting in a caspase dependent apoptosis of tumor cells [41]. Another example of a tumor suppressive function of Notch receptors has been reported in pancreatic ductal adenocarcinoma (PDAC). A mouse model described the oncogenic K-ras mutation in PDAC and simultaneous deletion of Notch1 in the pancreas leaded to increased tumor progression suggesting a tumor suppressive role of Notch in this cancer type [42]. There are also reports suggesting tumor suppressive functions of Notch1 in the mouse skin [43].
In general the function of the Notch signaling pathways in human cancer is difficult to predict and is highly context dependent. This is highlighted by different functions of the same receptor type in different cancer types. Notch3 for example has been reported to induce senescence and p21 expression in a variety of human cell lines including MCF-7 and HT144 showing the involvement of Notch 3 in regulation of senescence and tumor suppression [44]. On the other hand there are also reports suggesting a oncogenic role of Notch 3 in ovarian cancer by up regulation of Pdx1 which is a known a proto-oncogene in hematopoietic malignancy [45]. Another contributing aspect to the highly context dependent nature of the Notch signaling pathway are reports showing oncogenic and tumor suppressive functions of different Notch receptors in the same tumor type. In embryonal brain tumor cell lines expression of constitutively active Notch1 is showing tumor suppressive functions while constitutively active Notch2 shows tumor promoting functions [3].
The highly context dependent function of Notch signaling in cancer emphasizes the sensitivity of the signaling pathway to minor changes of the involved regulatory network. Investigations of different mutations in the protein-protein binding region of CSL revealed that one mutation can differently affect the regulation of the target gene expression depending on the promoter environment and the used NICD in vitro [46]. However understanding the cause of the specific outcome of the Notch signaling could ultimately lead to increased efficiency of the cancer therapy but still requires a lot of effort reveal all underlying mechanisms.

1.2 Epithelial-mesenchymal transition

The epithelial-mesenchymal transition (EMT) is a biological process that induces a phenotypical switch from an epithelial to a mesenchymal cell type. This change is accompanied by shedding of cell-cell and cell-matrix interactions, enhanced migratory capacity and invasiveness as well as elevated resistance to apoptosis, which all together, results in degradation of the underlying basement membrane and the formation of a mesenchymal cell that migrates away from the epithelial layer from which they originated (Figure 6) [47].

The molecular hallmark of this process is the down regulation of the cell-cell adhesion molecule E-cadherin and the up regulation of a number of mesenchymal markers, such as N-cadherin, Vimentin, and Fibronectin. These molecular alterations affect the phenotype of the cells and lead to the loss of their basal-apical polarity which results in a shift to a more spindle-shaped morphology [48].

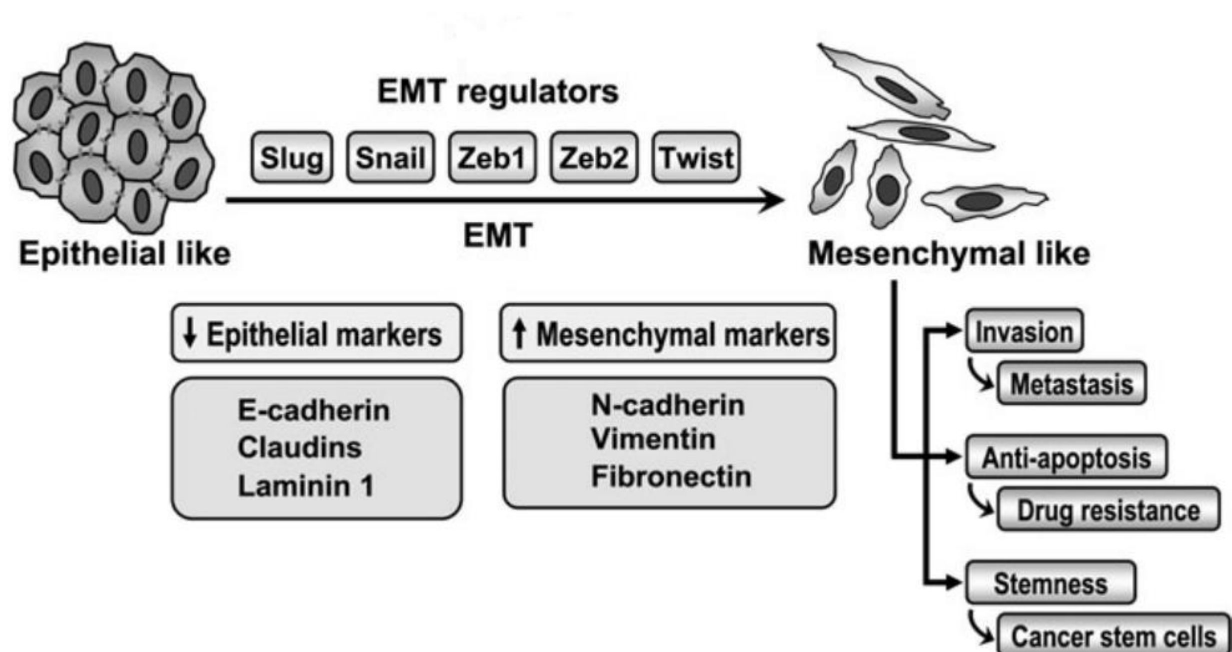


Figure 6: Overview of the epithelial-mesenchymal transition (EMT). *Cell exhibiting the epithelial phenotype undergo a complex alteration of their gene expression pattern which leads to a phenotypic switch resulting in a mesenchymal like cell. This change is induced by EMT regulators and leads to the suppression of epithelial markers and the up regulation of mesenchymal markers which ultimately results in increased invasiveness, elevated resistance to apoptosis as well as cancer stem cell like properties. [adapted from [49]]*

1.2.1 EMT, cancer progression and metastasis

Under normal physiological settings the EMT process has a central role in embryogenesis and wound healing and is therefore essential for the development and tissue regeneration of metazoan [50]. Most notably EMT is required for gastrulation and neural crest formation but is also involved in the development of organs like the cardiac heart valves, the skeletal muscle and the palate [51].

Beside its physiological roles the EMT is also involved in cancer progression and metastasis formation. In order to disseminate, epithelial cancer cells must undergo EMT to gain invasiveness necessary to leave the primary tumor mass. These mesenchymal cells are then capable of intravasation. After transportation through the circulation the cells undergo extravasation at a distant tissue and form micrometastases, which ultimately lead to metastasis formation. Interestingly the colonies at distant sites resemble the primary tumor from which they arose and therefore no longer exhibit the mesenchymal phenotypes that can be observed at the invasive front of primary tumors. In order to regain their initial phenotype as well as the proliferative capacity required for secondary tumor formation the metastasizing cancer cells must undergo a reversion of the EMT, the so called mesenchymal-epithelial transition (MET) (Figure 7) [47, 52]. The epithelial mesenchymal transition has been observed as part of the progression of many cancer types including but not limited to ovarian, breast and colon cancer. Generally the EMT that can be observed during cancer progression is presumably caused by reactivation of developmental signaling pathways during tumor formation. This hypothesis is strengthened by the fact that many of the EMT regulators, which are involved in the developmental EMT processes, show altered expression patterns in cancer cells and also show the expected correlation with features of the EMT process [51]. The melanoma precursors, the melanocytes, arise from the neural crest and do not belong to the epithelial lineage [53]. Therefore melanocytes cannot undergo classical EMT. However, cell-cell contact mediated by members of the cadherin family are important for the communication with keratinocytes and thus also for the regulation of differentiation and proliferation of the melanocytes [54]. Therefore the loss of E-cadherin expression during melanoma progression represents an EMT-like change that results in a highly mobile and invasive phenotype [55, 56].

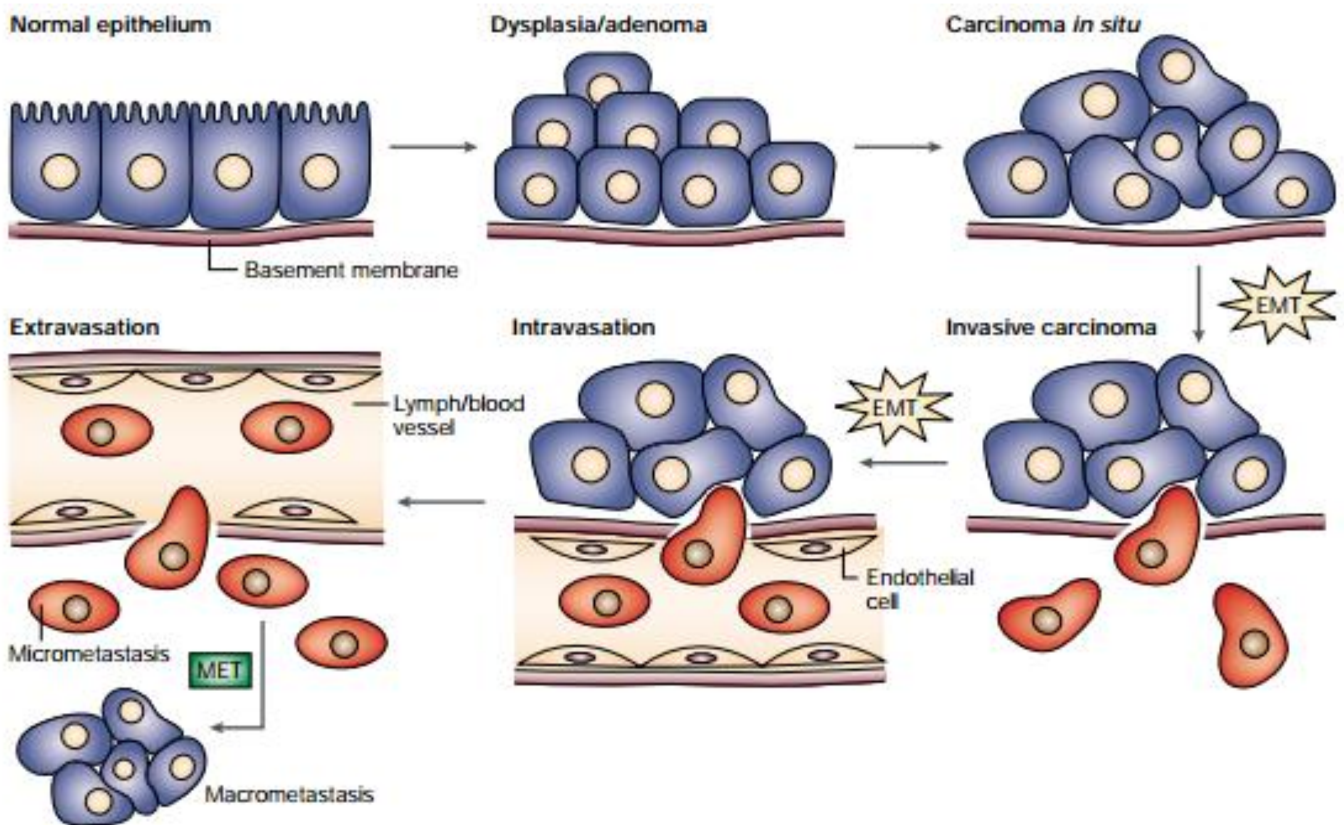


***Figure 7: Progression of metastasizing carcinoma cells**. Transformation of normal epithelial cells by epigenetic changes and genetic alterations can lead to adenoma and/or carcinoma formation. These carcinoma cells can then undergo epithelial-mesenchymal transition (EMT) and disseminate through the circulation. The mesenchymal like carcinoma cells leave the primary tumor mass and can intravasate into lymph or blood vessels allowing them to be passively transported to distant organs, were they leave the circulation through extravasation. These micrometastatses can then form a new carcinoma through a mesenchymal—epithelial transition (MET). [52]*

1.2.2 Transcription factors and other markers involved in EMT

The regulation of EMT is a complex process that involves multiple signaling pathways including Notch, Wnt, TGFβ and Hedgehog. Considering the complexity and number of signaling events and the possibility of cross talk between these pathways there are a high number of transcription factors involved [48]. Many of these transcription factors are E-box binding proteins that repress E-cadherin transcription either directly or indirectly. Members include the zinc finger transcription factors Snail, Slug, ZEB1 and SIP1, as well as basic helix–loop–helix transcription factors like E12/47 and Twist1 [57].

The zinc finger transcription factor Slug

The epithelial-mesenchymal transition regulator (EMTR) Slug (Snai2) belongs to the conserved family of Snail transcription factors which are known to play an important role during embryonic development [58]. Structurally the Snail family members share a conserved C-terminal domain while harboring a variable N-terminal domain. The C-terminal domain consists of five C2H2 type zinc finger domains that are responsible for DNA binding and repressor activity of the transcription factor [49]. The typical target sequence of the Slug protein is the conserved E-box sequence 5-CAGGTG-3 [59]. The less conserved N-terminal domain includes the SNAG domain that is present in all Snail family members and is required for the repressive function. The SNAG domain of Slug interacts with the co-repressors CtBP-1. CtBP-1 then recruits histone deacetylase which in turn leads to the suppression of target genes [60]. Furthermore the N-terminal domain of Slug includes a so called Slug motive which can only be found in Slug and not Snail1. This Slug domain is like the SNAG domain required for the repressor activity of the Slug and is involved in the recruitment of the co-repressor CtBP-1 [61]. The modular structure of Slug is summarized in Figure 8.

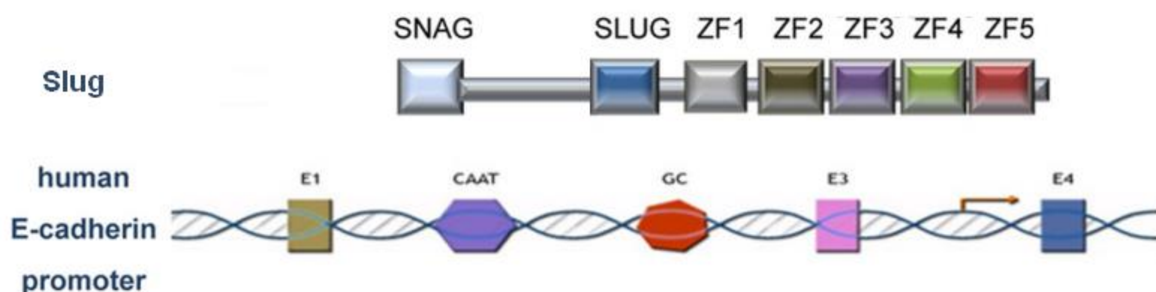


Figure 8: Schematic representation of the Slug protein and the human E-cadherin promoter. *The SNAG and SLUG domain are required for the recruitment of the co-repressor while the zinc finger domains (ZF1-5) are required for the sequence specific DNA binding. It has been shown that especially ZF3 and ZF4 are essential for the active repressor function. E1, E3 and E4 indicate the potential Slug binding sites. [adapted from [7]]*

The first identified Slug target gene was the epithelial marker E-cadherin. Binding of Slug to the E-cadherin promoter leads to the repression of gene activity and therefore is involved in the induction of the EMT process. Furthermore Slug has an activating effect on mesenchymal markers like Vimentin, Fibronectin, and N-cadherin.
This activation seems to be mediated by an indirect mechanism but is not well understood yet [7]. Slug knockout mice are viable but show a depigmentation of the body as well as the feet and tails suggesting an important role of Slug in melanocyte stem cells. Additionally Slug overexpressing transgenic mice develop mesenchymal carcinomas highlighting a potential oncogenic function of Slug [49].

The bHLH transcription factor Twist1

Another EMTR that has a central role in promoting EMT is the transcription factor Twist1. Twist1 has a molecular weight of 21 kDa (202 amino acids) and belongs to the basic helix loop helix (bHLH) transcription factor family. It forms dimers with the helices of the bHLH domain and binds to the conserved hexanucleotide sequence CATATG, the so called Nde1-Ebox, with the basic part of the same domain [62]. Unlike other bHLH transcription factors Twist1 is able to form both functional homo and heterodimers while a typical heterodimer partner include the E2A encoded transcription factors E12 as well as the bHLH transcription factor Hand2 [63, 64]. The balance between the Twist1/Twist1 (T/T) homodimers and the Twist1/E12 (T/E) is very important for the function of the Twist mediated regulation of target genes because both complex types exhibit different regulatory properties and are involved in the regulation of different gene sets. Indeed the different complexes can even have opposed effects as shown during Drosophila mesoderm development [65]. Ratio between the T/T and the T/E complexes is dependent on the protein Id1. Id1 is a HLH protein that has no basic domain and therefore dimers with Id1 are unable to bind DNA. Id1 has a higher affinity to bind E12 which reduces the available amount of the binding partner required for T/E complex formation, ultimately resulting in a shift towards the T/T homodimer [62].

2 Materials and Methods

2.1 Cell culture

The primary human melanoma cell line WM35, and the metastatic cell lines WM9, WM164 as well as stably transduced Notch4 overexpressing cell lines (kindly provided by Mr. Ehsan Bonyadi Rad) were grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO), supplemented with 2% bovine fetal serum (PPA Pasching Austria) and 2% L- glutamine (PPA Pasching Austria). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were harvested for the individual experiments after washing with phosphate buffered saline (pH 7.4) (Gibco Life Technologies Carlsbad, CA) using trypsin to detach the adherent cells. The cell suspension was then centrifuged for 5 minutes at 1000 rpm and the cell pellet was stored at -20°C or directly used for further analysis.

2.2 Bacterial transformation and plasmid isolation

The plasmids pCMV6-Hey1 pCMV6-Hey2 and pCMV6-XL5 were purchased from OriGene Technologies (Rockville, MD) while the plasmids pGL3-PromSlug, pGL3-PromSlugMut pGL3-PromTwist1 and pGL3-PromTwist1Mut were purchased from GenScript (Piscataway, NJ). 10 ng of the respective Plasmids were mixed with 100 µL Subcloning Efficiency™ DH5α™ Competent *E.coli* Cells (Invitrogen Life Technologies Carlsbad, CA) and incubated on ice for 30 minutes. Following the incubation period the mixture was heat shocked in a water bath (42°C) for 45 seconds and again incubated on ice for 10 minutes. Afterwards the cells were supplemented with 900 µL S.O.C. Medium (Invitrogen Life Technologies Carlsbad, CA) and incubated at 37°C for 1 hour with 225 rpm shaking. The transformed cells were then centrifuged (13000 rpm 1 minute), the supernatant reduced to a final volume of 100 µL and plated on LB-agar plates containing ampicillin (100 µg/mL). The agar plates were then incubated at 37°C for at least 12 hours. Single colonies of *E.coli* DH5α cells were suspended in 10 mL of LB medium supplemented with 100 µg/mL ampicillin and incubated at 37°C for 12-15 hours. These cells were then centrifuged at 3000 rpm for 3 minutes and used for plasmid isolation using the

QIAprep® Miniprep kit (QIAGEN, Hilden, GER) following the manufacturers protocol (May 2012).

2.3 Plasmid and siRNA transfection

siRNAs targeting mRNAs of Hey1 (SMARTpool: ON-TARGETplus HEY1 siRNA), Hey2 (SMARTpool: ON-TARGETplus HEY2 siRNA) and Notch4 (SMARTpool: ON-TARGETplus NOTCH4 siRNA) were purchased from Thermo Scientific/Dharmacon (Lafayette, CO). Control siRNA was purchased from QIAGEN (Hilden, GER). 150 pmol siRNAs or 2 µg of plasmid DNA were transfected into cells seeded in 6- well-plates using Lipofectamine 2000 reagent (Life Technologies Carlsbad, CA) according to the manufacturer's protocol (July 2006). Cells were harvested for mRNA analysis or Western Blot analyses 48 or 56 hours after transfection.

2.4 Protein concentration measurement: Bradford assay

To determine the protein concentration 3 µL of the protein solution was added to 1 mL of a 1:5 diluted Bradford Protein Assay reagent (BioRad, Hercules, CA) and incubated for 4 minutes at room temperature. The resulting color change was then determined using a photometer (Eppendorf BioPhotometer) at a wavelength of 595 nm. Pure diluted Bradford reagent was used as a blank.

2.5 Immunoblotting

Whole cell lysates were generated using RIPA buffer (Sigma-Aldrich St. Louis, MO) supplemented with 1% protease inhibitor cocktail (Active Motive Carlsbad, CA). The protein concentration was measured by the Bradford Protein Assay as described above. 15 µg of protein were then loaded on a 10% SDS-polyacrylamide gel and run at 110 V for 75 minutes. Following SDS-PAGE, protein was transferred to a polyvinylidene difluoride membrane (Millipore, Billerica MA) at 350 mA for 90 minutes and probed for the protein of interest with the specific antibodies shown in Table 1. Blots were blocked using 5% milk powder solution for 1 hour at room temperature and then incubated overnight at 4°C with the primary antibody. The peroxidase conjugated secondary antibody was then applied and incubated for 3 hours at room

temperature. Washing between the different steps was carried out using TRIS buffered saline supplemented with 1% Tween 20 (Merck Readington Township, NJ). Proteins were visualized using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Pittsburgh, PA) or Amersham ECL Western Blotting Detection Reagent (GE Healthcare Pittsburgh, PA) and exposed to X-ray film (Kodak). The membranes were stripped by incubating with Roti®-Free Stripping-Puffer (Roth Karlsruhe GER) for 45 minutes in a glass vessel at 50°C. After stripping, the membrane was thoroughly washed using TRIS buffered saline supplemented with 1% Tween 20 (Merck, Readington Township, NJ). Stripping and re-blotted was performed as required in the individual experiment. The resulting Western blots were scanned using a Chemi Doc TM XRS Universal Hood (Bio-Rad, Hercules, CA) and quantified using the ImageJ software. All bands were quantified in relation to the beta actin loading control.

Table 1: Western Blot antibodies

Primary antibody			**Secondary antibody**		
Target Protein	**Company**	**Dilution**	**Target**	**Company**	**Dilution**
Notch4	Cell signaling	1:2000	Rabbit anti mouse	Dako	1:1000
Slug	Santa Cruz	1:200	Donkey anti goat	Santa Cruz	1:4000
Twist1	Abcam	1:1000	Rabbit anti mouse	Dako	1:1000
β-Actin	Sigma	1:10000	Goat anti rabbit	Santa Cruz	1:4000
Hey1	Abcam	1:1000	Rabbit anti mouse	Dako	1:1000
Hey1	Abcam	1:1000	Goat anti rabbit	Santa Cruz	1:4000
Hey2	Abcam	1:1000	Goat anti rabbit	Santa Cruz	1:4000

2.6 Quantitative real-time RT-PCR

For mRNA analysis, RNA was isolated using the RNeasy kit from QIAGEN (Hilden, GER) following the manufacturer's protocol. The cDNA was generated using the First Strand cDNA Synthesis Kit from Thermo Scientific (Waltham, MA) using 2 µg mRNA as template for the random hexamer primed synthesis. Following reverse transcription, qPCR was performed using the gene specific primer together with Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX (Invitrogen Life Technologies Carlsbad, CA) Master Mix. Primers for Slug (QT00044128) and Twist1 (QT00011956) were purchased from QIAGEN (Hilden, GER). The Actin primers were synthesized by Ingenetix (Vienna AUT) according to the following sequence:

Actin FW: CCACACTGTGCCCATCTACG

Actin Rev: AGGATCTTCATGAGGTAGTCAGTCAG

Amplifications were performed on a 7900HT Real-Time PCR Analyzer (Applied Biosystems, Foster City, CA) using relative quantification with beta actin as reference gene and the ΔΔCT calculation for quantification. Each gene of interest has been measured in three replicates.

2.7 Electrophoretic mobility shift assay (EMSA)

Nuclear lysates of Notch4 overexpressing cell lines were generated using a Nuclear Extract Kit (Active Motive, Carlsbad, Ca) following the manufacturer's protocol. 10 µg of nuclear protein lysate were mixed with Cy3 labeled double strand oligonucleotide sequences resembling E-boxes of the respective promoter regions (Table 2), binding buffer [25 mmol/L HEPES (ph 7.5), 4 mmol/L MgCl2, 1 mmol/L EDTA, 0,5% Nonident P40, 10% glycerol] and 1 µg of poly (dI-dC) and incubated for 45 minutes at 37°C. After incubation the reaction mixtures were loaded on a 6% native polyacrylamide gel and run at 120 V for 80 minutes and analyzed using a Bio-Rad Molecular Imager FX to detect the retained Cy3 labeled DNA. Following the native PAGE the protein/DNA complexes were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica MA) at 350 mA for 90 minutes and probed for Hey1 or Hey2 using specific antibodies shown in Table 1 following the same procedure used for immunoblotting.

Table 2: EMSA sequences

Slug E-Box1	
WT	Mut
5'AATAAAC**CACCTG**AAAGTAT 3'	5'AATAAAC**ATCCTA**AAAGTAT 3'
Slug E-Box2	
WT	Mut
5'CCTCCAG**CACCTG**TTAGAAA 3'	5'CCTCCAG**ATCCTA**TTAGAAA 3'
Twist1 E-Box1	
WT	Mut
5'GCATTGC**CAGCTG**TTAGGGC 3'	5'GCATTGC**ATGCTA**TTAGGGC 3'
Twist1 E-Box2	
WT	Mut
5' GAACAGC**CACGTG**GCCTGCC 3'	5' GAACAGC**ATCGTA**GCCTGCC 3'

2.8 Luciferase assay

Hey1 or Hey2 mediated regulation of the Slug or Twist1 promoter activity was determined by co-transfection of pCMV6-XL5, pCMV6-Hey1 or pCMV6-Hey2 together with empty pGL3-basic, pGL3-PromSlug, pGL3PromSlugMut or pGL3-PromTwist1, pGL3PromTwist1Mut (complete sequences shown in the Section 7 Appendix) and a β-galactosidase reporter vector using Lipofectamine 2000 reagent (Life Technologies Carlsbad, CA) according to the manufacturer's protocol (July 2006). Cells were washed, harvested and probed for their luciferase and β-galactosidase activity using the ONE-Glo™ Luciferase Assay System (Promega, Madison, WI) and the Beta-Glo® Assay System (Promega, Madison, WI) following the manufacturers protocols for the respective assays. Detection of luminescence intensity was performed using the LUMIstar Omega luminometer (BMG Labtech, Offenburg, Germany). The luciferase activity was normalized to the β-galactosidase activity to ensure equal transfection efficiency.

3 Objective and Aims

In epithelial cancers EMT is a very well characterized process for the invasive capacity of malignant cells. The conversion of a mesenchymal phenotype into an epithelial phenotype, the MET process, for the development of metastases is still enigmatic but important to understand for the design of proper treatments. In melanoma knowledge about MET is sparse. Notch mediated signaling potentially regulating EMT regulators sets the base for the rationale to study these in the context of a presumed influence on EMT or MET transition.

Aims:

1. Investigating the relationship between Notch4 and the epithelial-mesenchymal transition regulators Slug and Twist1
2. Elaborating the involvement of Hey proteins in the regulation of EMTRs

4 Results

4.1 Notch4 suppresses the EMTRs Slug and Twist1 in human melanoma cell lines

Preliminary data showed that human melanoma cell lines stably transduced with a lenti-vector construct expressing the N4ICD (pLNCX-N4ICD) resulted in a strong repression of the EMTRs Slug and Twist1 at the protein level compared to empty vector transduced cells (data not shown).

In order to confirm these data, the effects of Notch4 overexpression on mRNA levels of the investigated EMTRs were assessed using quantitative real-time RT-PCR. In accordance with the preliminary results Notch4 overexpressing cell lines showed a significant decrease of Slug and Twist1 mRNA levels compared to empty vector transduced cell lines (Figure 9A).

Based on these findings Notch4 was silenced using sequence specific transiently transduced short interference RNA (siRNA) and the effects of these knockdown experiments were investigated on protein and mRNA level by immunoblotting and quantitative real-time RT-PCR respectively. Corresponding with the results of the overexpression experiments, knockdown of Notch4 resulted in an increase of Slug and Twist1 at protein and mRNA levels in all investigated cell lines (Figure 9B,C,D).

These in vitro experiments suggest that Notch4 is a negative regulator of the EMTRs Slug and Twist1 in melanoma.

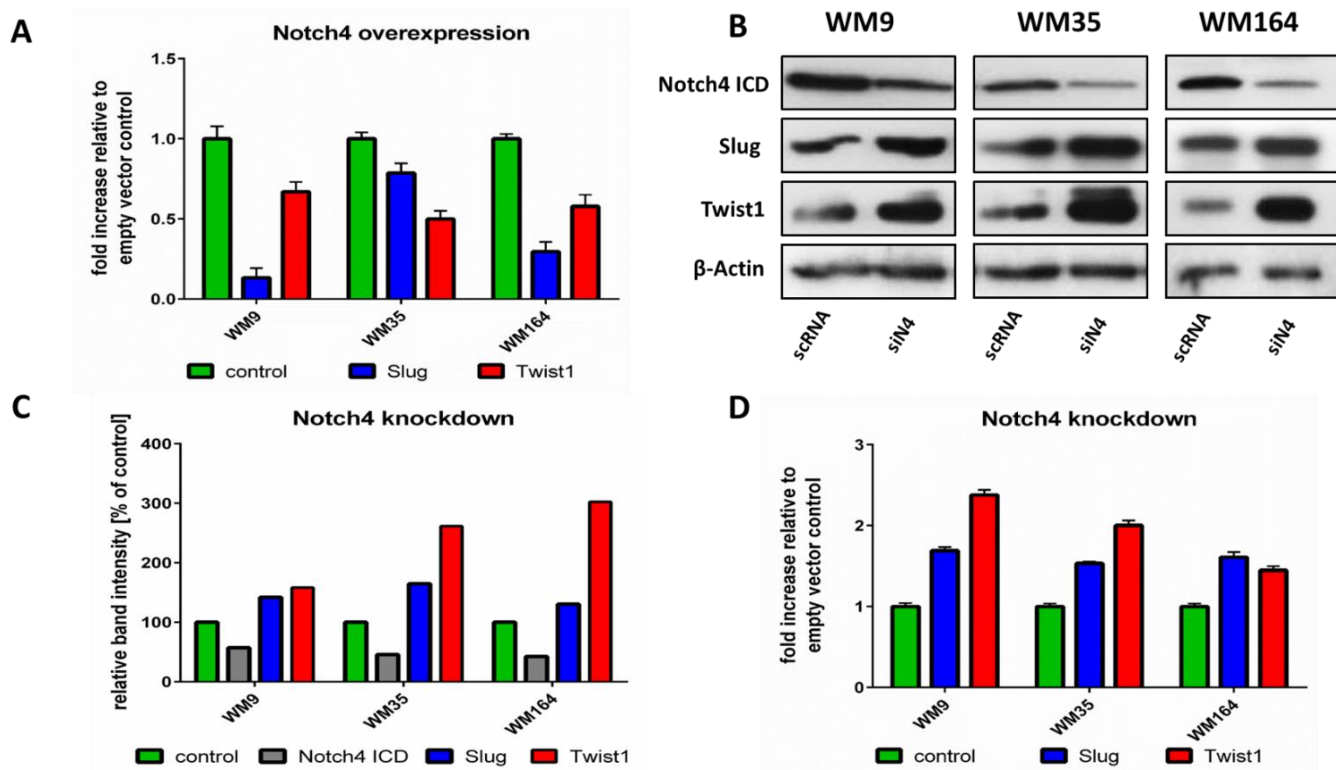


Figure 9: Notch4 is a suppressor of the EMTRs Slug and Twist1. *A, mRNA was extracted from cells stably transduced with a lenti-vector construct expressing the Notch4 intracellular domain (N4ICD) or empty vector transduced cells and analyzed using quantitative real-time RT-PCR for the expression levels of Slug and Twist1. mRNA expression levels were normalized to empty vector control and blotted as fold increase in a graph. The error bars represent standard deviations from the mean. B, Whole cell lysates of cells transfected with specific siRNA targeting Notch4 or unspecific scrambled RNA (scRNA) were subject to immunoblotting with specific antibodies for Notch4, Slug, Twist1 and β-Actin which was used as loading control. C, Semiquantitative analysis of the siN4 immunoblots was performed using ImageJ software. The band intensity's were normalized to the scRNA control and blotted as graphs. D, mRNA was extracted from cells transfected with specific siRNA targeting Notch4 or unspecific scrambled RNA (scRNA) and analyzed using quantitative real-time RT-PCR for the expression levels of Slug and Twist1. mRNA expression levels were normalized to scRNA control and blotted as fold increase in a graph. The error bars represent standard deviations from the mean.*

4.2 The Notch target gene Hey1 suppresses Slug and Twist1 expression

During the canonical Notch signaling the target gene transcription is usually activated through the formation of an complex consisting of CSL, the Notch intracellular domain, and the transcriptional activator Mastermind [11]. This type of signal transduction is unable to explain a repressor function of Notch4 that has been observed in the overexpression and knockdown experiments. However one of the best characterized Notch target genes constitutes transcriptional repressors of the Hey family [27].

In order to investigate a possible regulatory effect of the Hey family member Hey1 on the EMTRs, knockdown experiments using specific siRNA targeting Hey1 were performed and subsequently subject to immunoblotting and quantitative real-time RT-PCR. Indeed the knockdown of Hey1 resulted in an up regulation of Slug and Twist1 on protein (Figure 10A, B) and mRNA level (Figure 10C).
To confirm these results melanoma cells previously transiently transfected with plasmids expressing Hey1 or empty vector plasmids were subjected to immunoblotting and quantitative real-time RT-PCR. In accordance with the siRNA silencing experiments the overexpression of Hey1 resulted in the suppression of the EMTRs on both, the protein (Figure 10D,E) and the mRNA level (Figure 10F). These results suggest that Hey1 indeed is able to negatively regulate the expression of the epithelial mesenchymal transition regulators Slug and Twist1 in melanoma.

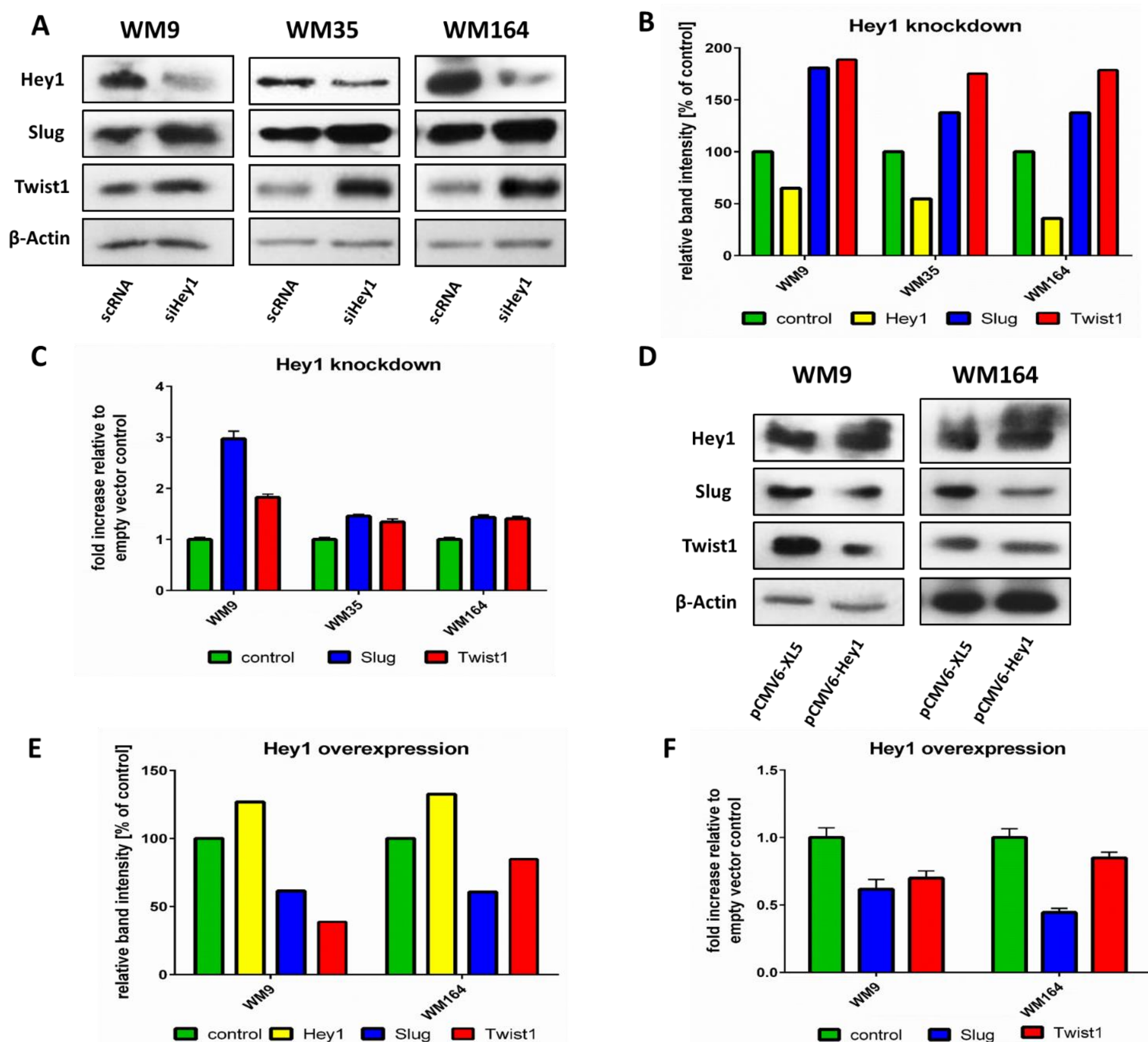


Figure 10: Hey1 is a suppressor of Slug and Twist1. *A, Whole cell lysates of cells transfected with specific siRNA targeting Hey1 or unspecific scrambled RNA (scRNA) were subject to immunoblotting with specific antibodies for Hey1, Slug, Twist1 and β-Actin which was used as loading control. B, Semiquantitative analysis of the siHey1 immunoblots was performed using ImageJ software. The band intensity's were normalized to the scRNA control and blotted as graphs. C, mRNA was extracted from cells transfected with specific siRNA targeting Hey1 or unspecific scrambled RNA (scRNA) and analyzed using quantitative rea- time RT-PCR for the expression levels of Slug and Twist1. mRNA expression levels were normalized to scRNA control and blotted as fold increase in a graph. The error bars represent standard deviations from the mean. D, Whole cell lysates of cells transfected with plasmids expressing Hey1 or empty vector control plasmids were subject to immunoblotting with specific antibodies for Hey1, Slug, Twist1 and β-Actin which was used as loading control. E, Semiquantitative analysis of the Hey1 overexpression immunoblots was performed using ImageJ software. The band intensity's were normalized to the empty vector control and blotted as graphs. F, mRNA was extracted from cells transfected with plasmids expressing Hey1 or empty vector control plasmids and analyzed using quantitative real-time RT-PCR for the expression levels of Slug and Twist1. mRNA expression levels were normalized to empty vector control and blotted as fold increase in a graph. The error bars represent standard deviations from the mean.*

4.3 The Hey family members Hey1 and Hey2 are up regulated after Notch4 overexpression

To test whether the Hey family members are up regulated in Notch4 overexpressing cell lines, whole cell lysates were investigated by immunoblotting (Figure 11A,B). As expected and in line with the literature Hey1 and Hey2 are up regulated in Notch4 overexpressing cells compared to empty vector transduced control cells. These results confirm that the regulatory effect of Notch4 on Slug and Twist1 could indeed be mediated by the Hey family of transcription factors.

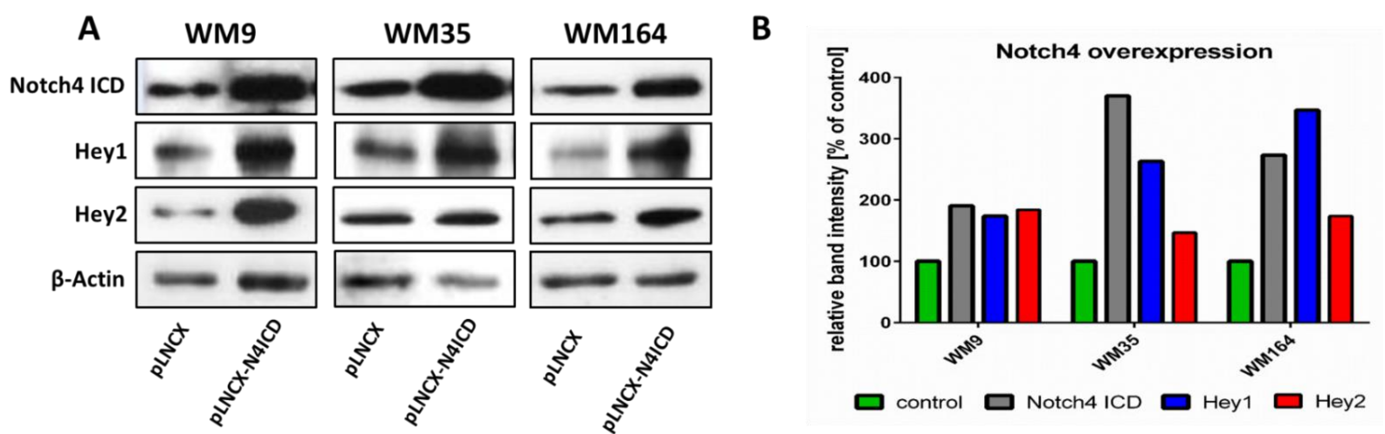


Figure 11: Notch4 induced up regulation of Hey1 and Hey2. *A, Whole cell lysates of cells stably transduced with a lenti-vector constructs expressing the Notch4 intracellular domain (N4ICD) or empty vector transduced cells were subject to immunoblotting with specific antibodies for Notch4 ICD, Hey1, Hey2 and β-Actin which was used as loading control. B, Semiquantitative analysis of the shown blots was performed using ImageJ software. The band intensity's were normalized to the empty vector control and blotted as graphs.*

4.4 The transcription factor Hey2 suppresses Slug but not Twist1 expression

Based on the finding that Hey1 is involved in the regulation of Slug and Twist1 the experiments were extended to examine the role of Hey2 in the Notch4 mediated regulation of the investigated EMTRs. Transient silencing experiments of Hey2 followed by immunoblotting revealed that knockdown of Hey2 resulted in an increased protein level of Slug while the protein level of Twist1 was unchanged (Figure 12A,B). To verify these results Hey2 silenced cells were probed by quantitative real-time RT-PCR (Figure 12C). These experiments confirmed that Slug is regulated by Hey2 on the transcriptional level. However Twist mRNA levels were also increased following the Hey2 knockdown.

To complement these findings Hey2 was transiently overexpressed and subject to immunoblotting (Figure 12D,E) and quantitative real-time RT-PCR (Figure 12F). The immunoblotting confirmed that overexpression of Hey2 results in a repression of Slug while not affecting the Twist1 protein level. In line with the protein levels, the mRNA analysis showed a significant suppressive effect of Hey2 on Slug. However the Twist1 mRNA level was again slightly reduced.

Taken together these results suggest that in contrast to Hey1, Hey2 down regulates Slug but not Twist1.

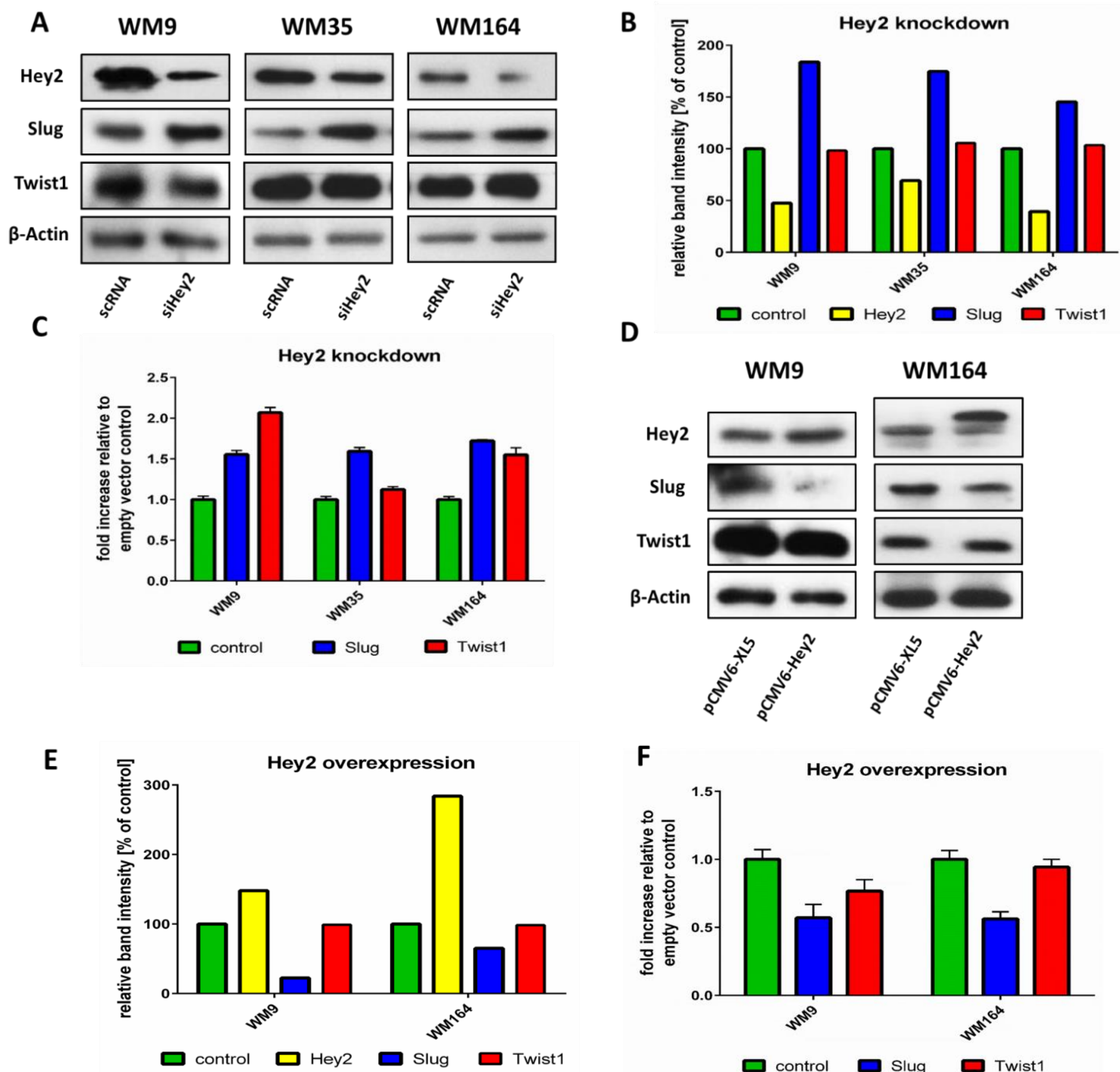


Figure 12: Hey2 is a suppressor of Slug but not Twist1. *A, Whole cell lysates of cells transfected with specific siRNA targeting Hey2 or unspecific scrambled RNA (scRNA) were subject to immunoblotting with specific antibodies for Hey2, Slug, Twist1 and β-Actin which was used as loading control. B, Semiquantitative analysis of siHey2 immunoblots was performed using ImageJ software. The band intensity's were normalized to scRNA control and blotted as graphs. C, mRNA was extracted from cells transfected with specific siRNA targeting Hey2 or unspecific scrambled RNA (scRNA) and analyzed using quantitative real-time RT-PCR for the expression levels of Slug and Twist1. mRNA expression levels were normalized to scRNA control and blotted as fold increase in a graph. The error bars represent standard deviations from the mean. D, Whole cell lysates of cells transfected with plasmids expressing Hey2 or empty vector control plasmids were subject to immunoblotting with specific antibodies for Hey2, Slug, Twist1 and β-Actin which was used as loading control. E, Semiquantitative analysis of the Hey2 overexpressing immunoblots was performed using ImageJ software. The band intensity's were normalized to the empty vector control and blotted as graphs. F, mRNA was extracted from cells transfected with plasmids expressing Hey2 or empty vector control plasmids and analyzed using quantitative real-time RT-PCR for the expression levels of Slug and Twist1. mRNA expression levels were normalized to empty vector control and blotted as fold increase in a graph. The error bars represent standard deviations from the mean.*

4.5 The Hey protein family members Hey1 and Hey2 act downstream of Notch4

The observation of Hey1 and Hey2 regulating Slug and Twist1 raised the question if they both act downstream of Notch4 or if there are other mechanisms involved in the regulation of these EMTRs by Notch4.

In order to investigate the hierarchy of Notch4 and the Hey proteins a double transfection was performed. Melanoma cells were simultaneously transiently transfected with specific siRNA targeting Notch4 and plasmids encoding Hey1 or Hey2 respectively. Immunoblotting of whole cell lysates of these co-transfected cells overexpressing Hey1 showed that Slug and Twist1 were suppressed despite a significant decrease in Notch4 protein level (Figure 13A,B).

In accordance with the previous Hey2 experiments, co-transfection of siN4 and pCMV6-Hey2 showed the suppression of Slug by Hey2. Furthermore Twist1, which has been shown to be unaffected by Hey2, is increased in the Notch4 silenced and Hey2 overexpressing cells which can be attributed to the down regulation of Notch4 (Figure 13C,D).

Altogether the co-transfection experiments indicate that the regulation of Slug and Twist1 by Notch4 is mediated by Hey1 and Hey2 and that these transcription factors overrule Notch4 in the regulation of the investigated EMTRs.

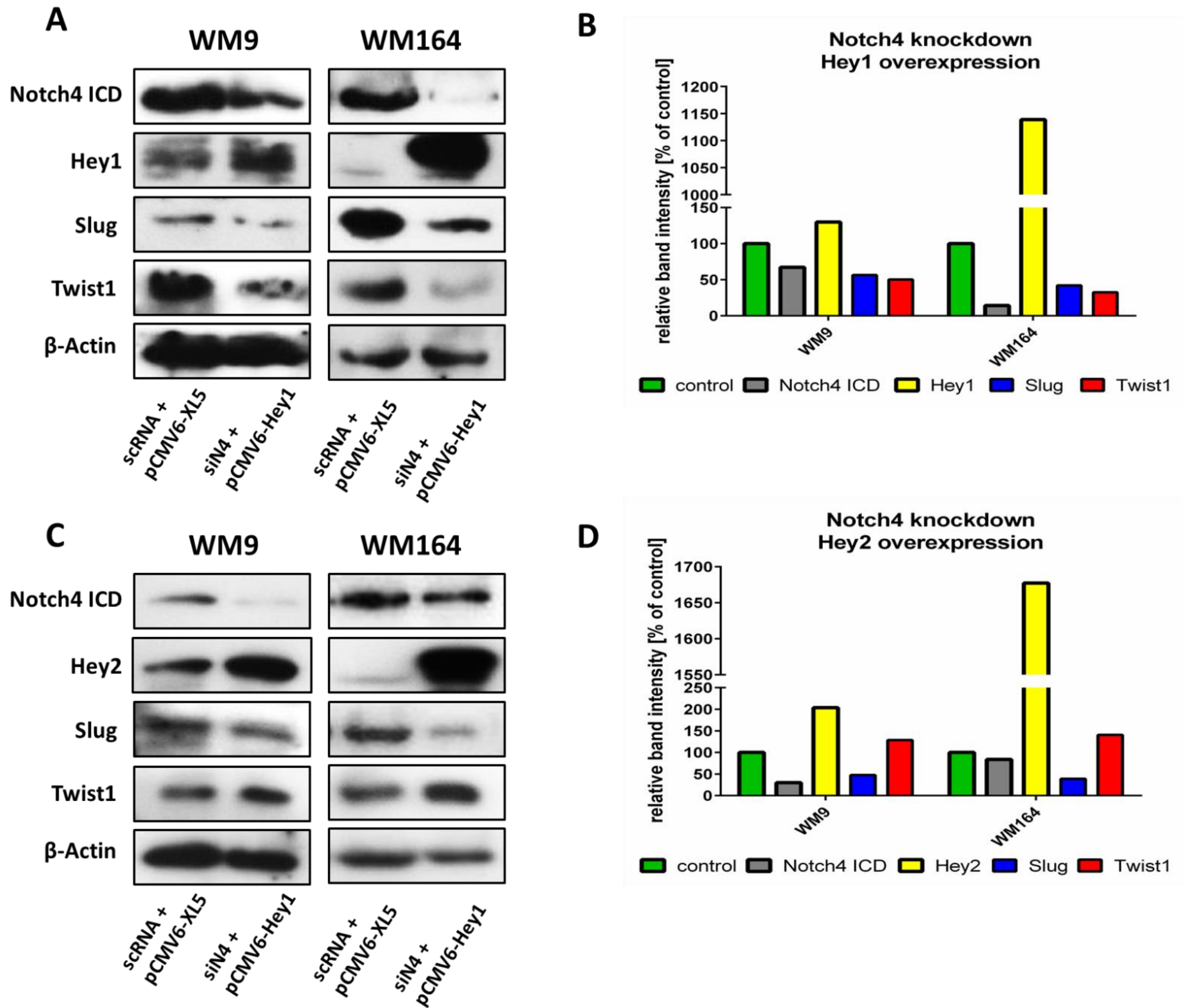


Figure 13: Hey1 and Hey2 act downstream of Notch4. *A, whole cell lysates of cells transiently co-transfected with specific siRNA targeting Notch4 and plasmids encoding Hey1 were subject to immunoblotting with specific antibodies for Notch4 ICD, Hey1, Slug, Twist1 and β-Actin which was used as loading control. B, semiquantitative analysis of the siN4 Hey1 overexpression immunoblots was performed using ImageJ software. The band intensity's were normalized to the empty vector control and blotted as graphs. C, whole cell lysates of cells transiently co-transfected with specific siRNA targeting Notch4 and plasmids encoding Hey2 were subject to immunoblotting with specific antibodies for Notch4 ICD, Hey2, Slug, Twist1 and β-Actin which was used as loading control. D, semiquantitative analysis of the siN4 Hey2 overexpression immunoblots was performed using ImageJ software. The band intensity's were normalized to the empty vector control and blotted as graphs.*

4.6 The Hey protein family members Hey1 and Hey2 bind directly to Slug and Twist1 promoter.

After identifying Hey1 and Hey2 as the mediators of the Notch4 driven regulation of Slug and Twist1 the question remained whether these basic helix loop helix transcription factors are directly or indirectly regulating the EMTRs.

Analysis of the promoter regions of Slug and Twist1 revealed the presence of E-boxes which are potential Hey1/2 binding sites (Figure 14A). To test whether Hey proteins bind to the identified E-Boxes in the respective promoter regions electrophoretic mobility shift assays (EMSA) were performed whereupon two of the identified E-boxes were chosen randomly and are subsequently referred to as E-Box1 and E-Box2 (Figure 14A). The EMSAs confirmed that both Hey1 and Hey2 are capable of binding to the used DNA fragments while mutations of the E-Box sequences in these fragments strongly reduced the binding capacity (Figure 14B,C).

To verify that direct DNA binding is indeed affecting the promoter activity a luciferase reporter assay was performed to assess changes of the promoter activity following Hey protein overexpression. The luciferase assay showed that the Slug promoter activity is strongly suppressed by overexpression of Hey1 or Hey2 and that this suppressive effect is partially abrogated by mutation of the identified E-Box sequences (Figure 15A). The reporter assay of the Twist1 promoter regions also showed the suppressive effect of Hey1 and Hey2 overexpression (Figure 15B). This again confirms that Hey2 seems to increase the Twist1 transcription which, however, does not translate to increased protein levels.

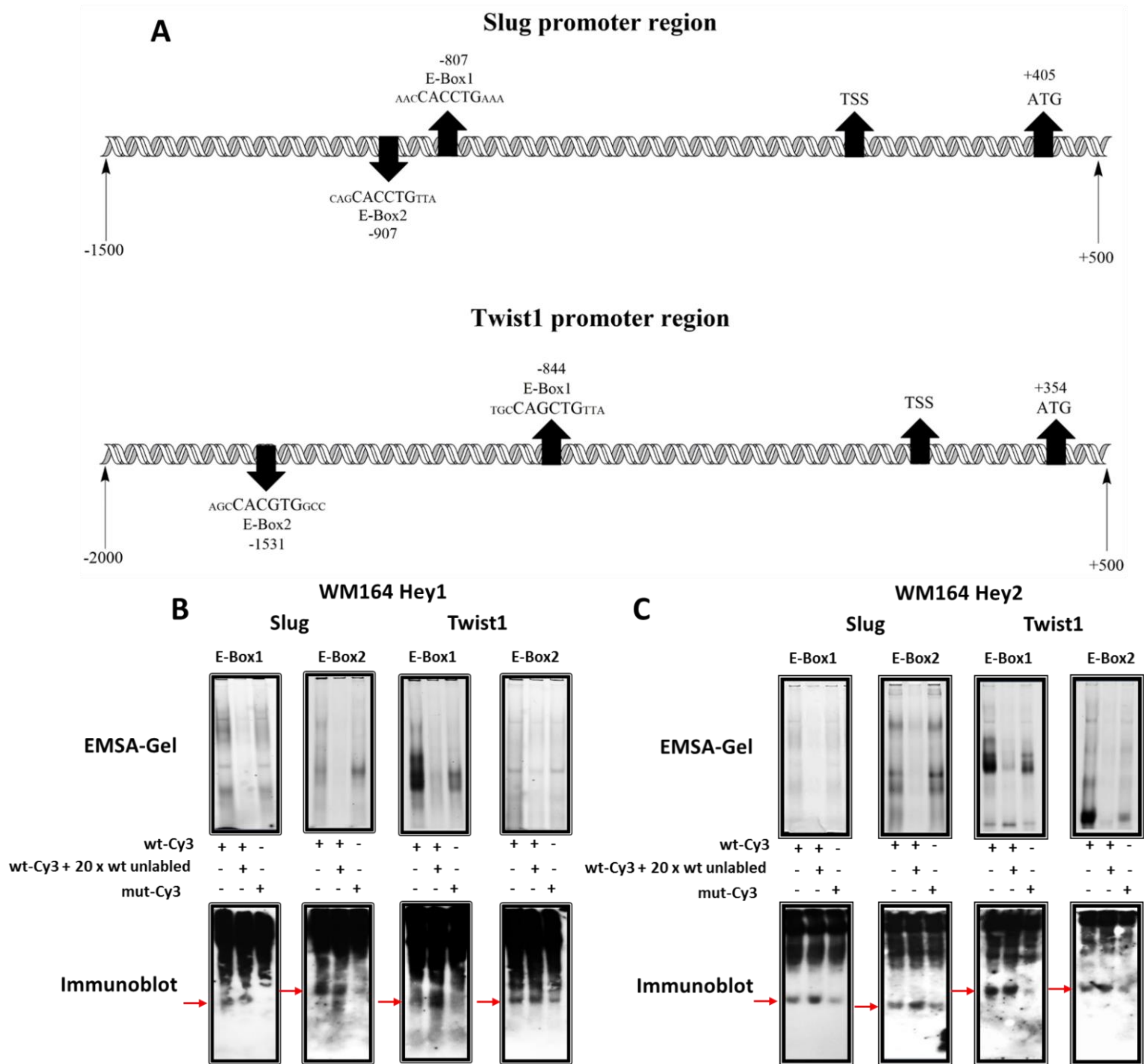
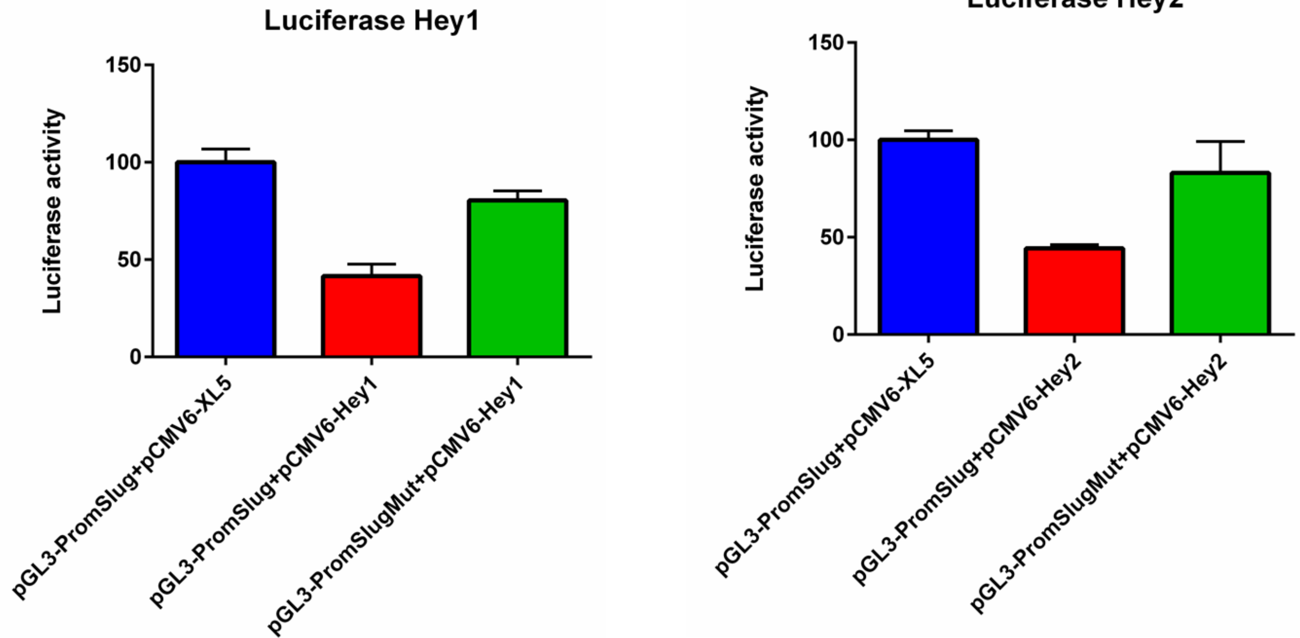


Figure 14: Electrophoretic mobility shift assay. *A, Scheme of the Slug promoter region from -1500 to +500 and the Twist1 promoter region from -2000 to +500 relative to the transcription start (TSS). Identified and randomly chosen potential Hey1 and Hey2 binding E-Boxes as well as the translational start (ATG) are indicated. B and C, 15 µg of nuclear extract of WM164 melanoma cells were incubated with 20 nt fragments of Cy3 labeled wild type (wt), Cy3 labeled wild type together with 20 fold excess of unlabeled wild type or Cy3 labeled mutated E-box identified in the Slug and Twist1 promoter regions for 45 minutes at 37°C. The DNA-protein complexes were separated by native PAGE (6%), the labeled DNA was detected using a Bio-Rad Molecular Imager FX (EMSA-Gels) and the complexes were subject to immunoblotting with specific antibodies for Hey1 or Hey2. Bands representing the Protein/DNA complexes are indicated by red arrows.*

A **Luciferase Slug Promoter**



B **Luciferase Twist1 Promoter**

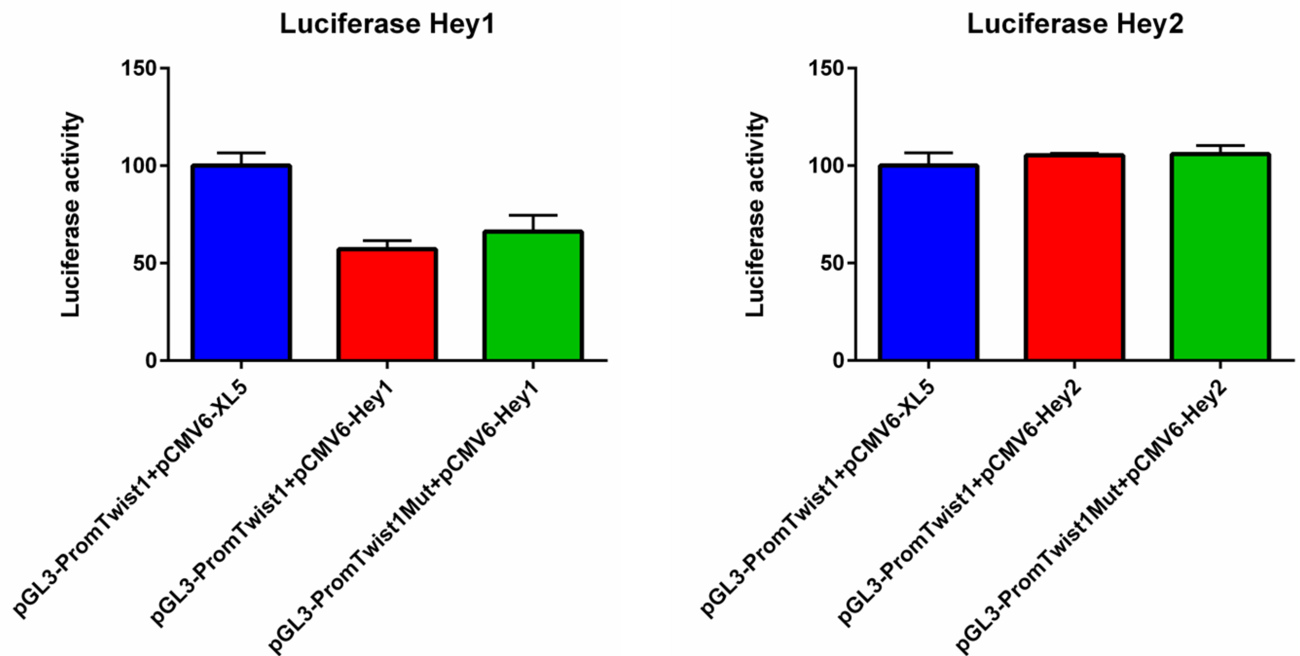


Figure 15: Luciferase assay of Slug and Twist1 promoter regions. *A, WM164 cells were co-transfected with either pGL3-Basic + pCMV6-XL5, pGL3-Basic + pCVMV6-Hey1 or pCMV6-Hey2, pGL3-PromSlug +PCMV6-XL5 or pCMV6-Hey1 or pCMV6-Hey2, pGL3-PromSlugMut + pCMV6-Hey1 or pCMV6 Hey2 and a β-galactosidase reporter vector using Lipofectamine 2000. Luciferase activity was measured 48 hours post transfection and normalized to the β-galactosidase activity to account for differences in transfection efficiency and cell confluency.*

5 Discussion

Considering the fundamental roles of the Notch signaling pathway during the embryogenic development it is no surprise that Notch is involved in oncogenesis and tumor progression [1]. In the development of skin cancer the role of aberrant Notch signaling is not clearly defined. Nicolas et al. showed that Notch1 deficiency in the mouse skin caused the development of basal-cell carcinoma–like tumors suggesting a tumor suppressive role of Notch in the skin [43]. On the other hand Notch1 has been shown to be one of the drivers of melanoma progression by activating mitogen-activated protein kinase (MAPK) and Akt pathway, which ultimately leads to up regulation of N-cadherin [66]. Additionally Hardy et al. suggested that Notch4 is involved in the manifestation of an aggressive melanoma phenotype by regulating the embryonic morphogen Nodal [5].

This study shows that Notch4 is involved in the regulation of the epithelial-mesenchymal transition in melanoma. The regulatory effect is mediated indirectly by the known Notch target genes Hey1 and Hey2, which suppress the transcription of the epithelial-mesenchymal transition regulators Slug and Twist1. Furthermore it shows that the transcription factors Hey1 and Hey2 are directly binding to E-boxes of the promoter region and are directly regulating the promoter activities of both investigated EMTRs.

The previously reported importance of Slug for the metastasis formation of melanoma [67] and the direct activation of Slug by the transcription factor Zeb1, which together synergistically repress E-cadherin expression [57], imply that the finding of a repressive function of Notch4 on the transcription level of Slug can further lead to strong and diverse downstream effects in response to Notch4 alterations. Recent work of Fenouille et al. highlighted the importance of Slug for the regulation of cellular functions. It was shown that knockdown of Slug attenuated the SPARC induced increase of invasiveness and migratory capacity [56]. Twist1, just like Slug, has also been reported to be a direct transcriptional repressor of E-cadherin [45]. The central role of Twist1 in tumor development has been described in a large variety of cancer types. In melanoma specifically it has been reported that 80% of the investigated samples show an increased Twist1 expression compared to normal healthy tissue.

The same study further showed that Twist1 and mitogenic oncoproteins such as Ras and ErbB2 cooperatively act to down regulate epithelial markers such as E-cadherin or Claudin-7 while inducing mesenchymal markers such as Vimentin or N-cadherin [68].

The Notch mediated regulation of Slug has already been described in breast cancer cells. Leong et al. showed that Jagged-1 mediated Notch1 activation in breast cancer leads to activation of Slug in a CSL dependent manner [69]. This observation is in contrast to regulatory mechanism of Slug by Notch4 described in this study. However unpublished data generated by Mag. Ehsan Bonyadi Rad showed direct binding of CSL to the Slug promoter region. Silencing experiments of CSL (performed during the project laboratory) further revealed that it acts as a suppressor of the Slug transcription, suggesting that activated Notch was not binding to CSL in order to activate gene transcription in these experimental conditions. Rather than directly activating Slug expression the current study shows that Notch4 is suppressing Slug indirectly through Hey1 and Hey2 activation which highlights the highly context dependent action of the Notch signaling pathway. It is noteworthy that the same study reporting Notch1 as a direct activator of Slug also describes a positive correlation of Notch activity, Slug expression and the expression of the Hey family of transcription factors [69]. This in turn suggests that not only the Notch signaling itself but also the downstream signaling is highly context dependent.

There are also reports describing the regulation of Twist1 by Notch1. However compared to Slug, only indirect regulatory mechanisms have been reported. Hsu et al. showed that Twist1 promoter activity was regulated by Notch1 through STAT3 and not CSL dependent mechanism and that the other Notch receptors had no effect on Twist1 expression levels in gastric cancer [70]. The current study shows another indirect negative relationship between Nocth4 and Twist1. However unpublished data generated by Mag. Ehsan Bonyadi Rad showed direct binding of CSL o the Twist1 promoter suggesting the possibility of a direct activation of Twist1 by Notch. Like in the case of Slug, silencing experiments of CSL (performed during the project laboratory) indicated a suppressive function of CSL on the Twist1 transcriptional activity. Taken together the presented data identifies Slug and Twist1 as target genes of the Notch signaling pathway and show an indirect suppressive effect of Notch4 on the transcription of both EMTRs (Figure 16). Nevertheless the mechanisms that

decide whether Notch acts as an activator or suppressor or the Notch/CSL complex formation are highly context dependent and still need to be examined in more detail.

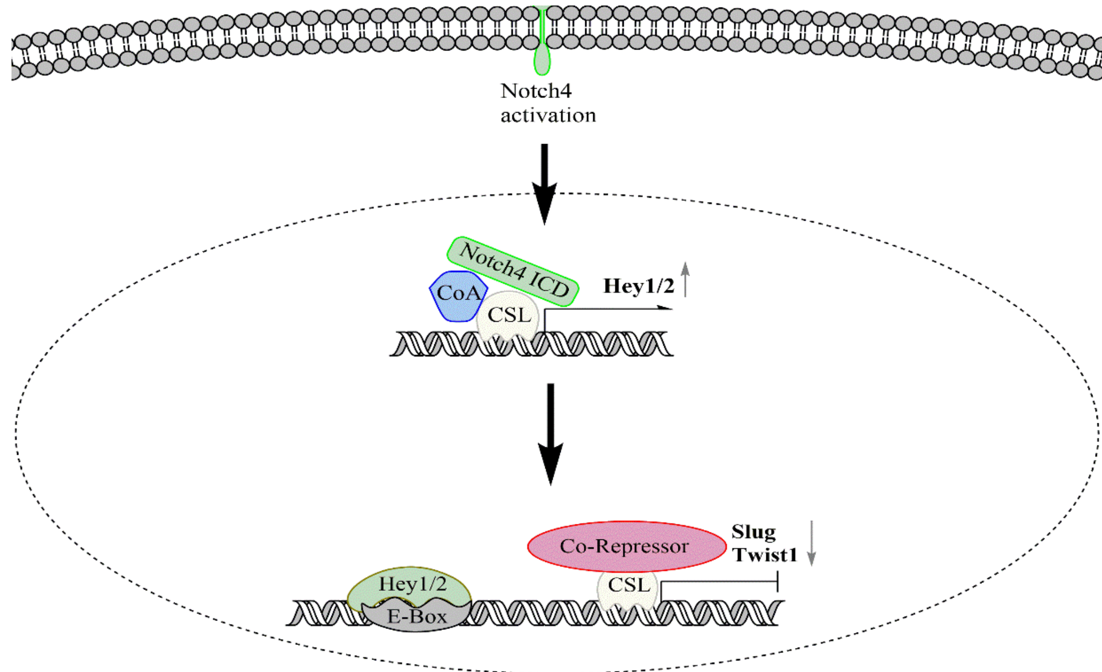


Figure 16: Summary of Notch4 mediated regulation of Slug and Twist1. *Notch4 activation is followed by a CSL dependent up regulation of HEY1 and Hey2. The Hey proteins then bind to E-box sequences in the Slug and Twist1 promoter regions and suppress the transcription. CSL is also present in the Slug and Twist1 promoters but is not activated by Notch4 thus also acts as a transcriptional repressor.*

In the view of the many reports of pro-oncogenic functions of Slug and Twist1 in many cancer types including melanoma, the current study suggests that Notch4 can act as a tumor suppressor. This tumor suppressive function could especially block metastasis formation by promoting a mesenchymal to epithelial transition thus inhibiting the dissemination of the primary tumor mass, trapping the cancer cells in a low invasive and low migratory epithelial state. However it has to be noted that one of the possible consequences of the mesenchymal to epithelial transition, re-expression of E-cadherin, has also been linked to cell survival at metastatic sites in breast carcinoma [71]. Moreover, several studies reported the involvement of MET in promoting metastatic colonization (reviewed in [72]) which raises the question if the observed effect of Notch4 is a part of the metastasis formation in melanoma. In order to answer this and other remaining questions regarding the selectivity of the Notch signaling, further investigations are necessary to increase the understanding of cancer progression and metastasis formation, which could lead to the development of more efficient therapeutic strategies.

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7 Appendix

Sequences of the Luciferase fragments. E-Boxes are highlighted in yellow, mutated E-Boxes are highlighted in red.

Twist1 Luciferase fragment

AGTCTTGTTTACACCTCGCTGGAGAAATAACACTCGCCCTCACTTCTCCCAAAAAGCTGAACCCTT
CAGTCGGCCCAAGCAGCTCCACACCCTGAGGTTTCCAAGACCAAAGCTGCGAGTCTCAGCAGGG
AACAGCCACGTGGCCTGCCTGCGCCTCGCCTGGGCTCTTGCCTTCAGCTTGAGATATCTGCAGC
CGCGAACCTTGCTCCAGCCCAGAAAGGGGCGCTTTGCTCAATTAATTGTTCCCGCCGGCGAGTC
CGTACTGAGAAGCCCATGAGCGGACCTTATGTGCAGGGTACTCCAGCGCGGTGCACAAAACTCG
TCGCCCCCAAACGCTGCCCCCACCCCAACACTGTGTACTGACTCCAGCTTTTTACTTTGCCATGT
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CAAAAGATGGGCTTCTTATCCAAGGCGACTTCCTTTCTGGTTCACCAACTTTGCTGCTTCCAGTTT
GCCAGGATCTACATTAACACCCTCTTTGGGGCTCTTCGTTTTAACTTACAGACAGAAATGCTTAAA
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AAATTCCCACTTCACTCTCTTACAAGCTGGCCTTTCAAGGTCACAATGCGGAGCCTAATTTGGGG
GTGGGGATGAAATGGCCACAGGGTCTCTCCCTTGGGTTGGCATTGCCAGCTGTTAGGGCCGCAG
CAAAGGCGCTGCGCTGCCCCCCTCTGGCTCTGCTGCCTTTCCCATGGACTGGGTTTCCTTCCAC
CGAAGAGTGAACTTCTGCCTCTTTCGAGCACCTTCCGAGGCGTAGTCCTTTGGATGTTGGGGAG
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CGAATTGTTAGACCCCGAGGAAGGGAGGTGGGACGGGGGAGGGGGACTGGAAAGCGGAAACTT
TCCTATAAAACTTCGAAAAGTCCCTCCTCCTCACGTCAGGCCAATGACACTGCTGCCCCCAAACT
TTCCGCCTGCACGGAGGTATAAGAGCCTCCAAGTCTGCAGCTCTCGCCCAACTCCCAGACACC

Mutated Twist1 Luciferase fragment

AGTCTTGTTTACACCTCGCTGGAGAAATAACACTCGCCCTCACTTCTCCCAAAAAGCTGAACCCTT
CAGTCGGCCCAAGCAGCTCCACACCCTGAGGTTTCCAAGACCAAAGCTGCGAGTCTCAGCAGGG
AACAGCATCGTAGCCTGCCTGCGCCTCGCCTGGGCTCTTGCCTTCAGCTTGAGATATCTGCAGC
CGCGAACCTTGCTCCAGCCCAGAAAGGGGCGCTTTGCTCAATTAATTGTTCCCGCCGGCGAGTC
CGTACTGAGAAGCCCATGAGCGGACCTTATGTGCAGGGTACTCCAGCGCGGTGCACAAAACTCG
TCGCCCCCAAACGCTGCCCCCACCCCAACACTGTGTACTGACTCCAGCTTTTTACTTTGCCATGT
AAGGGATGGACCTGAAACGGTTATTTTACCTCAATTCATTTCAAAAAGGAAACAAGTATGGCATTG
CAAAAGATGGGCTTCTTATCCAAGGCGACTTCCTTTCTGGTTCACCAACTTTGCTGCTTCCAGTTT
GCCAGGATCTACATTAACACCCTCTTTGGGGCTCTTCGTTTTAACTTACAGACAGAAATGCTTAAA
ATGTTAGCGTATCCAAGCATTTGGAATTGGGGCTCACGAAGCCTAATTGTCCACTGGATGCCCTA
GATAGTGGGGGCTGGGGCGGGGGGGGTCTCAGAGCGGGCAGCCCCTATGTCTAGGCGCTATC
AAATTCCCACTTCACTCTCTTACAAGCTGGCCTTTCAAGGTCACAATGCGGAGCCTAATTTGGGG
GTGGGGATGAAATGGCCACAGGGTCTCTCCCTTGGGTTGGCATTGCATGCTATTAGGGCCGCAG
CAAAGGCGCTGCGCTGCCCCCCTCTGGCTCTGCTGCCTTTCCCATGGACTGGGTTTCCTTCCAC
CGAAGAGTGAACTTCTGCCTCTTTCGAGCACCTTCCGAGGCGTAGTCCTTTGGATGTTGGGGAG
CGTCAGACTGGGTCGTTGTAGAGGGGAAAGGAGGGCCCAGAAGGGCGAGAGAGCAGGCCGGG
ACGCAAATCCTCAGCCCCCGCGGCGCGGCCACGTCTTCAGAAACGCCCAGGACCTCCGGGCTG
GGCCGCCGCGGTTTGGCCTTTGGAACTCCAAGGGGTTCGTCTACCTGACCATTGGGTGGGCTCC
GCGGTTGACACTTTTCTTGGCATGCCCCCCCACCCCGCGCCACACCACCCCCCCAGCCCCAGCA
ATCCCAAATCGGCCCCACGGACCTAGAGGGCTCTTGGGCGAGATGAGACATCACCCACTGTGTA
GAAGCTGTTGCCATTGCTGCTGTCACAGCCACTCCGGATGGGGCTGCCACCGCGGCCAGGACA
GTCTCCTCCGACCGCTTCCTGGGCTGCGCTAGGGTTCGGGGGCGCTGCCCGCACGCTCCGGCG
GGGAAGGAAATCGCCCCGCGCCCGCCGGAGGAAGGCGACGGGGAGGGAAGGGGGAGGGCGG
CTAGGAGGCGGGTGGAGGGGCCGGCCGCCCGGGCCAGGTCGTTTTTGAATGGTTTGGGAGGA
CGAATTGTTAGACCCCGAGGAAGGGAGGTGGGACGGGGGAGGGGGACTGGAAAGCGGAAACTT
TCCTATAAAACTTCGAAAAGTCCCTCCTCCTCACGTCAGGCCAATGACACTGCTGCCCCCAAACT
TTCCGCCTGCACGGAGGTATAAGAGCCTCCAAGTCTGCAGCTCTCGCCCAACTCCCAGACACC

Slug Luciferase fragment

TGTAACAAAAATGTGTGTTTTGTGGGAAATGGAGTGAAAAGCAAGGAGGACTCCTGCTCTCATAA
ACCCAGGTGCCTACATCCGAACAAACCCTCACATAGAGTAGCAAGCTTAGAAAACTGCCTCTGGC
TTTTACTCCAGGTTCCAGTTTGTGTGTGGAGAAATCGAATATGTATTCATATTTTCCTCTCTCCTGC
AAGTACAGTTCCATTAGCATAAGGATTCCTTTGAATTATTTTCTCTGTTTGACATTTAAATTAGTGA
CTGTTGGAAGAAATAAGAATAATCATTGAAATTAGCTTAGGAAATCTGTGAGTGCCCCACCTCACC
CTCCAAACACACATACACTCTTGTTTTCTCATTCCAAGATTAAGAAGTTATGCAACCTGACAATGC
ACTTTTCTCTGACAAGTCTTGACATCACCACTGTTATTTTCTTTTTCACTTTTTTCCTCCAGCACCT
GTTAGAAACAAGAGTAGGGTGATCTTCATGTGAATTTGTTCTTTCCTTATTCTTTTAGCAAAAGATA
GGGATAAAAGTCTGCAATGGACAGAGATGCTTTAAAATATGCTTGAAATTTTATATTTAAAATAAAC
CACCTGAAAGTATTTTTAGATTGAAGACCATACACAAAATAAAGGTGTTTTTGCAGTTACTAGACT
GGGTTCTTAAAACTTTAATCAATCTATATTCATAATAATATCCTTCAATTTCTTTTTTAGCATTATACA
GGAAACTGGTAGATACTGAGATGGATTTTAATGGCTTTATACTGATGAAAATAGCACCACATAAAA
GCAGGGGAATATTAGAAATAAAAATAATTGTCTCTAAAGACCCATACAACCCTTTTTCCCATAAAAA
AAAAGATGCACTGTAATACATGAAAAGATAAGATCTCTTGTCAAAAGTGTGAGAGAATGTCCGGTG
GTTCCAAATGACAGTTACCTCTTGCCCCCCTTCTCTGCCAGAGTTCCTTTTTATCTTTGCAATCTTC
CAGTTCTTCCGATCAGCCTGCCTTTAGAGGGCTACAAAGCATTTCTTTCAAGCCACCATAGCTAAC
ACGGTGACATGAGTACTTAATTTGCACGCGGCCGCGCTGCCCCTGGCTTCGCGGAAGCCCTGAG
TAGCGCAGCGCCCTCGCCGCACGCAAGGCTGCAGTCCCGCTCCAGGCCAGAGTCCCAGGAGAG
CGTCCTCCGCGCTCACAGGCGCCTTTGTCTTCCCGCTTCCCCCTTCCTTTTTCAAAAGCCAAGAG
GTAATTATTTGGTCTTTGTGCAAGGCAAACCTCTCCAGATGCCACTTCCAAATATAGGCTCTCATT
AACACCAGAGGCTGGCCTGGTGTGGTGCAGGGCGGCCCTTCCTTCTCCTGGCGGACACTGTGT
CCCCGCGCGCTGGCGCTGCACCACATCTGGAAGCCAGGCGGGCAGGGCAGAGACCCCGGCTC
CTGCGCCCCTCCTAGCTCCCAGAGAGCGTGGATCGCGGGCGGGGCTCACCGAGCGAGGTTACC
TCTCTTGAAAATACTTAAACACTTTTTTTCCTCTCCACTGAAATCTCAAAAAACAGCCCATTTTGAA
CCAGAATAATTTAGTCTGACAACAGATTCTTCCTCTGTTCACAGCTGTCCCAGAGGGAGGAGCTG
AAATCTGAACCTCTCAGCTGTGATTGGATCTTTCTTGCAAAAGAGAGGAAAAAAAAACCCTCCCAG
CCAAAACGGGCTCAGTTCGTAAAGGAGCCGGGTGACTTCAGAGGCGCCGGCCCGTCCGTCTGC
CGCACCTGAGCACGGCCCCTGCCCGAGCCTGGCCCGCCGCGATGCTGTAGGGACCGCCGTGT
CCTCCCGCCGGACCGTT

Mutated Slug luciferase Fragment

TGTAACAAAAATGTGTGTTTTGTGGGAAATGGAGTGAAAAGCAAGGAGGACTCCTGCTCTCATAA
ACCATGGTACCTACATCCGAACAAACCCTCACATAGAGTAGCAAGCTTAGAAAACTGCCTCTGGC
TTTTACTCCAGGTTCCAGTTTGTGTGTGGAGAAATCGAATATGTATTCATATTTTCCTCTCTCCTGC
AAGTACAGTTCCATTAGCATAAGGATTCCTTTGAATTATTTTCTCTGTTTGACATTTAAATTAGTGA
CTGTTGGAAGAAATAAGAATAATCATTGAAATTAGCTTAGGAAATCTGTGAGTGCCCCACCTCACC
CTCCAAACACACATACACTCTTGTTTTCTCATTCCAAGATTAAGAAGTTATGCAACCTGACAATGC
ACTTTTCTCTGACAAGTCTTGACATCACCACTGTTATTTTCTTTTTCACTTTTTTCCTCCAG**ATCCTA**
TTAGAAACAAGAGTAGGGTGATCTTCATGTGAATTTGTTCTTTCCTTATTCTTTTAGCAAAAGATAG
GGATAAAAGTCTGCAATGGACAGAGATGCTTTAAAATATGCTTGAAATTTTATATTTAAAATAAAC**A**
TCCTAAAAGTATTTTTAGATTGAAGACCATACACAAAATAAAGGTGTTTTTGCAGTTACTAGACTGG
GTTCTTAAAACTTTAATCAATCTATATTCATAATAATATCCTTCAATTTCTTTTTTAGCATTATACAGG
AAACTGGTAGATACTGAGATGGATTTTAATGGCTTTATACTGATGAAAATAGCACCACATAAAAGC
AGGGGAATATTAGAAATAAAAATAATTGTCTCTAAAGACCCATACAACCCTTTTTCCCATAAAAAAA
AAGATGCACTGTAATACATGAAAAGATAAGATCTCTTGTCAAAAGTGTGAGAGAATGTCCGGTGG
TTCCAAATGACAGTTACCTCTTGCCCCCCTTCTCTGCCAGAGTTCCTTTTTATCTTTGCAATCTTCC
AGTTCTTCCGATCAGCCTGCCTTTAGAGGGCTACAAAGCATTTCTTTCAAGCCACCATAGCTAACA
CGGTGACATGAGTACTTAATTTGCACGCGGCCGCGCTGCCCCTGGCTTCGCGGAAGCCCTGAGT
AGCGCAGCGCCCTCGCCGCACGCAAGGCTGCAGTCCCGCTCCAGGCCAGAGTCCCAGGAGAG
CGTCCTCCGCGCTCACAGGCGCCTTTGTCTTCCCGCTTCCCCCTTCCTTTTTCAAAAGCCAAGAG
GTAATTATTTGGTCTTTGTGCAAGGCAAACCTCTCCAGATGCCACTTCCAAATATAGGCTCTCATT
AACACCAGAGGCTGGCCTGGTGTGGTGCAGGGCGGCCCTTCCTTCTCCTGGCGGACACTGTGT
CCCCGCGCGCTGGCGCTGCACCACATCTGGAAGCCAGGCGGGCAGGGCAGAGACCCCGGCTC
CTGCGCCCCTCCTAGCTCCCAGAGAGCGTGGATCGCGGGCGGGGCTCACCGAGCGAGGTTACC
TCTCTTGAAAATACTTAAACACTTTTTTTCCTCTCCACTGAAATCTCAAAAAACAGCCCATTTTGAA
CCAGAATAATTTAGTCTGACAACAGATTCTTCCTCTGTTCA**ATGCTA**TCCCAGAGGGAGGAGCTG
AAATCTGAACCTCT**ATGCTA**TGATTGGATCTTTCTTGCAAAAGAGAGGAAAAAAAAACCCTCCCAG
CCAAAACGGGCTCAGTTCGTAAAGGAGCCGGGTGACTTCAGAGGCGCCGGCCCGTCCGTCTGC
CG**ATCCTA**AGCACGGCCCCTGCCCGAGCCTGGCCCGCCGCGATGCTGTAGGGACCGCCGTGTC
CTCCCGCCGGACCGTT