Molecular Mechanisms Underlying the Oncogenic Function of SS18 and SSX

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I'm digging for fire
The Pixies

To my parents

Abstract

The *SS18* and *SSX* genes were initially identified based on their reoccurrence as fusion partners in synovial sarcoma. As a result of the specific chromosomal translocation t(X:18), the *SS18* gene from chromosome 18 becomes fused with members of the *SSX* gene family on the X chromosome resulting in the generation of a novel chimeric fusion gene *SS18-SSX*. The *SS18* gene encodes a ubiquitously expressed transcriptional activator, whereas the *SSX* gene encodes a transcriptional repressor whose expression is restricted to germ cells and numerous cancers. Thus, the resultant *SS18-SSX* fusion gene encodes a transcription factor with dual trans activation and repression properties; the expression of which is the initiating event of synovial sarcoma.

We present the findings that SSX, along with several other members of the CT-antigen family is expressed in mesenchymal stem cells and their expression is down regulated following differentiation. Knockdown of SSX could effectively impair cell migration, a phenotype associated with down regulation of MMP2 expression adding a functional role for SSX in stem and tumor cell migration. We further explored the potential oncogenic mechanism of SSX expression by investigating the *in vitro* and *in vivo* role of SSX in altering cell growth and cell cycle progression. Using an inducible siRNA system we show that knockdown of endogenously expressed SSX can inhibit cell proliferation and tumor growth of *in vivo* xenographs. We demonstrate that SS18-SSX induces p53 ubiquitination, degradation and prevents the transactivation of p53 target genes following the induction of a stress response. The negative effect on p53 function was attributed to the ability of SS18-SSX to stabilize HDM2. We developed this notion by studying the p53 pathway in synovial sarcoma cell lines expressing wild type p53. We show that these cells were defective for inducing p53 trans activation in response to genotoxic stress; however treatment with the HDM2 antagonist nutlin-3A can could effectively restore p53 trans activation and apoptosis, suggesting that targeting the p53-HDM2 auto regulatory loop may be of therapeutic benefit for synovial sarcoma.

List of Publications

I. Cronwright G, Le Blanc K, Gotherstrom C, **D'Arcy P**, Ehnman M, Brodin B.
Cancer/testis antigen expression in human mesenchymal stem cells: down-regulation of SSX impairs cell migration and matrix metalloproteinase 2 expression.
Cancer Res. 2005 Mar 15;65(6):2207-15

II. Maruwge W*, **D'Arcy P***, Brodin B.
SSX activates β-catenin transcriptional function and sustains tumor cell proliferation *in vitro* and *in vivo*.
Submitted
* Equal contribution

III. **D'Arcy P**, Maruwge W, Ryan B A, Brodin B.
The oncoprotein SS18-SSX1 promotes p53 ubiquitination and degradation by enhancing HDM2 stability.
Mol Cancer Res 2008. Jan 6(1)

IV. **D'Arcy P**, Ryan B A, Brodin B.
Reactivation of wild type p53 tumor function in synovial sarcomas by inhibition of p53-HDM2 interaction.
S*ubmitted*

Related Publications

Maruwge W, **D'Arcy P**, Folin A, Brnjic S, Wedje J, Davis A, Erlandsson F, Bergh J, Brodin B.
Preclinical studies of Sorafenib in sarcoma models

Submitted

Contents

Abbreviations

A	Adenine
aa	Amino Acid
ActD	Actinomycin D
AF10 (MLLT10)	Myleoid/lymphoid or mixed-lineage leukemia translocated to chr 10
Akt (PKB)	Protein kinase B
ARF	Alternative reading frame
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia mutated related
Bak	Bcl2 homologous antagonist/killer
Bax	B cell associated X protein
Bcl-2	B cell lymphoma 2
Bcl-X_L	Bcl-2 related protein X_L
BRG1	SWI/SNF related regulator of chromatin
BRM	Human Brama homologue
C	Cytosine
CBP	CREB binding protein
CDK	Cyclin-dependent protein kinase
cDNA	Complementary DNA
Chk1/2	Checkpoint kinase 1-2
c-myc	Cellular myelocytomatosis oncogene
CoAA	Co activator activator protein
CT antigens	Cancer testis antigens
DD	Divergent domain
Der	Derivative chromosome
DNA	Deoxyribonucleic acid
Dox	Doxycycline
Doxo	Doxorubicin
DSB	Double strand breaks
Eto	Etoposide
FISH	Fluorescence in situ hybridization
G	Guanine
G	Glycine
GADD45	Growth arrest and DNA damage inducible 45
BRM1	SWI/SNF- related chromatin regulator
HDM2 (MDM2)	Human double minute 2 (Murine double minute 2)
IGF	Insulin like growth factor
IGF1R	Insulin like growth factor receptor
IGFBP	IGF binding proteins
K	Lysine
Kb	Kilo base
kDa	Kilo Dalton
KRAB	Krüppel associated box
MAPK	Mitogen activated protein kinase

MMP2	Matrix metalloproteinase 2
MSC	Mesenchymal stem cell
mRNA	Messenger RNA
P	Proline
PcG	Polycomb group
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
RT-PCR	Real time polymerase chain reaction
PI3K	Phosphatidylinositol 3' kinase
Puma	p53 up regulated modulator of apoptosis
Q	Glutamine
RAB3IP	RAB3 interacting protein
RNA	Ribonucleic acid
SH	Src homology
SIP/CoAA	SS18 interacting protein/co activator activator
siRNA	Small interfering RNA
SNH	SS18 N terminal homology domain
SS	Synovial sarcoma
SS18	Synovial sarcoma translocation chromosome 18
Ss18	SS18 murine homologue
SS18L1	SS18 Like 1
SS18L2	SS18 Like 2
SSX	Synovial sarcoma X chromosome breakpoint
SSXRD	SSX repression domain
SSX2IP	SSX2 interacting protein
SV40LT	Simian virus 40 large T antigen
SWI/SNF	Switch/sucrose non fermenting
SYT	Synovial sarcoma translocation
T	Thymine
Wt	Wild type
Ub	Ubiquitin
Utr	Untranslated region
XRCC4	X-ray cross complementation 4
Y	Tyrosine
YAC	Yeast artificial chromosome

Introduction

Cancer

Cells are the structural units of all living things. Each of us has trillions of cells which form a highly ordered ecosystem and as such are governed by a wide array of signals which control diverse cellular processes. The most fundamental characteristic of cells is their ability to reproduce themselves by dividing. The division of normal and healthy cells occurs in a regulated and systematic fashion such that cells divide only when required to do so. Cells which disobey normal growth controls are swiftly eliminated by inbuilt suicide programs. Failure to eliminate such cells when they arise can result in a cascade of genetic events ultimately resulting in the development of cancer. Although cancer is often referred to as a single condition, it actually consists of more than 100 different diseases which can affect different parts of the body. Despite the fact that they vary greatly in the site and tissue these diseases are typically characterized by uncontrolled growth and spread of abnormal cells. Even though the clinical manifestations of cancer are well documented the underlying molecular mechanisms are still relatively unknown. Recent discoveries in cancer deregulated pathways have raised the hope of developing treatments which target specific cancers on the molecular level.

Sarcomas and chromosome translocations

Sarcomas are a heterogeneous group of tumors derived from connective tissue. In comparison with other tumor types, sarcomas are relatively rare, accounting for approximately 1% of all diagnosed cases [1]. The etiology of these tumors is unknown and the vast majority of cases occur without known hereditary factors. A common feature in many types of sarcoma is the presence of cytogenetic anomalies called translocations. Translocations result in the genesis of abnormal genetic material through the illegitimate recombination of different chromosomes. The significance of translocations in cancer development has recently been underscored by a census which has shown that chromosome translocations are the most common mutation class accounting for over 75% of mutations in somatically mutated cancer cells [2]. A consequence of translocation is gene fusion which may result in the production of a chimeric protein. Since the breakpoints on each gene are usually located within intronic regions, the coding sequences of the genes are maintained in

the same reading frame allowing for the subsequent translation of a fusion protein with novel properties derived from both parent proteins. Initial studies on chromosomal translocations in sarcomas have identified numerous chimeric fusion genes generated by recurrent chromosomal abnormalities (Table 1). Since these solid tumours generally lack other cytogenetic abnormalities the chromosome translocation and the generation of a fusion gene is presumed to have an etiologic role in the early development of these tumours [3-6].

Table 1 *Chromosomal translocations with fusion genes in solid tumours*

Tumour	Translocation	Fusion gene
Ewing's sarcoma	t(11;22)(q24;q12)	*EWS-FLI1*
	t(21;22)(q22;q12)	*EWS-ERG*
	t(7;22)(p22;q12)	*EWS-ETV1*
	t(17;22)(q12;q12)	*EWS-E1AF*
Synovial Sarcoma	t(X;18)(p11;q11)	*SS18-SSX1*
		SS18-SSX2
		SS18-SSX4
Myxoid liposarcoma	t(12;16)(q13;p11)	*FUS-CHOP*
	t(12;22)(q13;q12)	*EWS-CHOP*
Aveolar sarcoma	t(2;13)(q35;q14)	*PAX3-FKHR*
Rhabdoidmyosarcoma	t(1;13)(q36;q14)	*PAX7-FKHR*
Clear cell sarcoma	t(12;22)(q13;q12)	*EWS-ATF1*
Dermatofibro-sarcoma	t(17;22)(q22;q13)	*COL1A/ PDGFB*
Desmoplastic small cell	t(11;22)(q13;q12)	*EWS-WT1*
Myxoid chondrosarcoma	t(9;22)(q22;q12)	*EWS-TEC*

Recurring translocations show strict specificity, where their presence is often found exclusively in a single tumor type The mechanism behind this specificity is still unclear; however one explanation for the strict connection to tumor type may be that the translocation and the resulting fusion gene may cause tumor development only when they occur in certain cell types, differential stages, tissue locations or microenvironments. Alternatively fusion genes may be able to induce differentiation programs and thereby differentiate cells into specific tumor types [3].

The history of *SS18* and *SSX*: A tale of two genes.

In 1986 several independent groups reported the identification of a reoccurring chromosomal abnormality present in synovial sarcoma [7-9]. Cytogenetic analysis of these tumors confirmed the presence of a specific chromosomal translocation between chromosome 18 and the X chromosome. Initial fluorescence in situ hybridization (FISH) analysis, using X-chromosome specific yeast artificial chromosome (YAC) probes identified two alternative and mutually exclusive Xp11.2 breakpoints in the vicinity of ornithine aminotransferase-like pseudo genes 1 and 2 (*OATL1* or *OATL2*) [10-13]. Subsequent molecular studies using YAC probes spanning the X chromosome breakpoint to screen a synovial sarcoma cDNA library lead to the isolation of a chimeric cDNA containing fragments of two novel genes *SS18* (previously *SYT*, *SSXT,* MGC116875) on chromosome 18 and *SSX* localized in the *OATL* pseudo gene cluster on the X chromosome. In accordance with the FISH results two different but closely related genes were found to be involved in fusions with *SS18; SSX1*, located in the *OATL1* region and *SSX2*, located in the *OATL2* region [14-17].
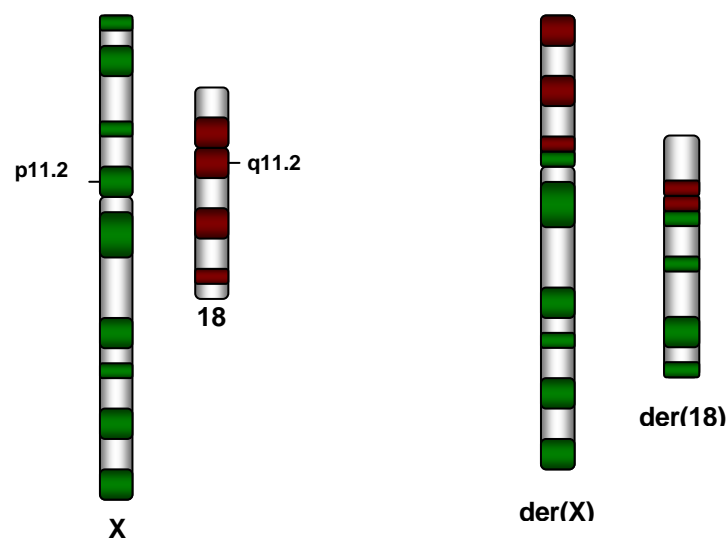

Figure 1.The reciprocal chromosomal translocation t(X:18) of synovial sarcoma.

Further molecular studies of the t(X;18) breakpoint has shown that the proximal portion of the *SS18* gene from chromosome 18 is disrupted and juxtaposed to the distal portion of either *SSX1* or *SSX2* genes on the X chromosome, in a mutually exclusive fashion [14, 16, 18].

SS18 has also been found fused to *SSX4* in synovial sarcoma, however the prevalence for this type of fusion is low with only four reported cases [19-21]. Like other soft tissue sarcomas, synovial sarcomas are often difficult to diagnose purely on histological and clinical grounds. Since the *SS18-SSX* fusion gene has been found in more than 95% of cases of synovial sarcoma, its detection is routinely used as a sensitive diagnostic test [13]. Compared with other types of sarcoma and carcinomas the number of chromosome aberrations per tumour is remarkably low. Secondary genetic aberrations have been found in only 50% of all synovial sarcoma cases, however none of these genetic aberrations have been shown to influence clinical outcome further supporting the notion that the sole chromosome translocation may be the essential event in synovial sarcoma development [22].

Clinical significance of alternative *SSX* fusions.

To date, only *SSX1*, *SSX2* and in rare cases, *SSX4* have been shown to take part in the synovial sarcoma chromosomal translocation [14, 19, 23]. It is still unknown why *SSX1*, *SSX2* and *SSX4* genes are the only members of the *SSX* family to be observed in chromosomal translocations, or why *SSX1* and *SSX2* predominate over *SSX4*. One hypothesis to why the *SS18-SSX4* translocation is rare may be related to the relatively low probability for *SSX4* rearrangements to occur or to the reduced oncogenic properties of the proteins they encode. Similarly the absence of *SSX3, SSX5-SSX9* fusions may be due to the localization of these genes in genomic regions that are less prone to rearrangements in synovial sarcoma precursor cells or to the reverse orientation of these genes on the X chromosome, hampering in frame fusions with *SS18* [24]. Genomic analysis of the intronic regions of *SS18-SSX* has shown the abundance of repetitive regions near the translocation breakpoint. A common feature in the cases investigated was the presence of sequences homologous to consensus topoisomerase II cleavage sites, at or near the breakpoints in both the *SS18* and *SSX* genes [25]. The pathological implications of the alternative *SS18-SSX* fusions suggest that the *SSX* subtype can influence clinical outcome. A correlation between fusion type and tissue subtype was shown where the majority of all biphasic synovial sarcomas carried *SS18-SS*X1 gene fusions whereas *SS18-SSX2* positive tumours were consistently found to be monophasic in nature [18].

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In comparison with *SS18-SSX1*, the occurrence of the *SS18-SSX2* fusion was associated with a statistically significant better metastasis free survival time [26, 27]. These results were later confirmed by a large multi centre study involving the retrospective analysis of 243 cases [22], however these results have also been disputed [28].

Synovial sarcoma: A tumor of myoblastic origin

In spite of its designation synovial sarcoma bears no physiological resemblance to synovial tissue [29]. The uncertainty of the tissue origin of synovial sarcoma has recently been addressed with a conditional knock in mouse model of synovial sarcoma which suggests that synovial sarcoma is derived from myoblastic pre cursor cells [30]. Conditional expression of human SS18-SSX2 in immature myoblast cells led to the occurrence of synovial sarcoma with 100% penetrance in mice models. Expression of SS18-SSX2 in more differentiated muscle cells induced myopathy in the absence of tumor induction whereas widespread expression of SS18-SSX2 during mouse embryogenesis resulted in lethality at E8.5. These results suggest that cells are only permissive to SS18-SSX induced tumorgenesis at specific developmental time points or differentiation stages.

SS18: the transcriptional activator

Located at position q11.2 on the long arm of chromosome 18, the *SS18* gene encodes a protein of 418 amino acids with a predicted molecular weight of 53 kDa [24]. Although SS18 does not show any overt homologies to any other known proteins, the protein contains several well characterized functional domains and displays an amino acid profile suggestive of a role in transcriptional activation [31]. The SS18 protein C terminal domain is rich in glutamine (19%), proline (16%), glycine (14%) and tyrosine (12%) and has been designated the QPGY domain to reflect the amino acid profile of this region [23]. The notion that SS18 functions as a transcriptional activator is supported by the finding that fusion of SS18 to a GAL4 DNA binding domain could activate the transcription of a luciferase reporter gene placed downstream of a GAL4 binding site [32, 33]. The importance of the QPGY domain in SS18 mediated trans activation was underscored by experiments with deletion QPGY mutants which displayed reduced transcriptional activation potential [32, 33].

A novel 54 amino acid N terminal domain designated the SNH domain was identified based on phylogenetic comparison. This domain is highly conserved between all species signifying an important regulatory role [32, 34, 35]. Deletion of the SNH domain enhances transcriptional by SS18 mutants, suggesting a potential function for this domain in regulating the transactivation properties of SS18 [32, 33, 36]. Since no consensus DNA binding domain has been identified, SS18 presumably functions as a transcriptional co activator via interactions with sequence specific DNA binding partners [37]. Deletional studies have determined that the SNH domain of SS18 is responsible for its nuclear localisation [37]. Since the N terminal of SS18 does not contain any consensus NLS it is presumed is retained in the nucleus through associations with a protein bearing a functional NLS.

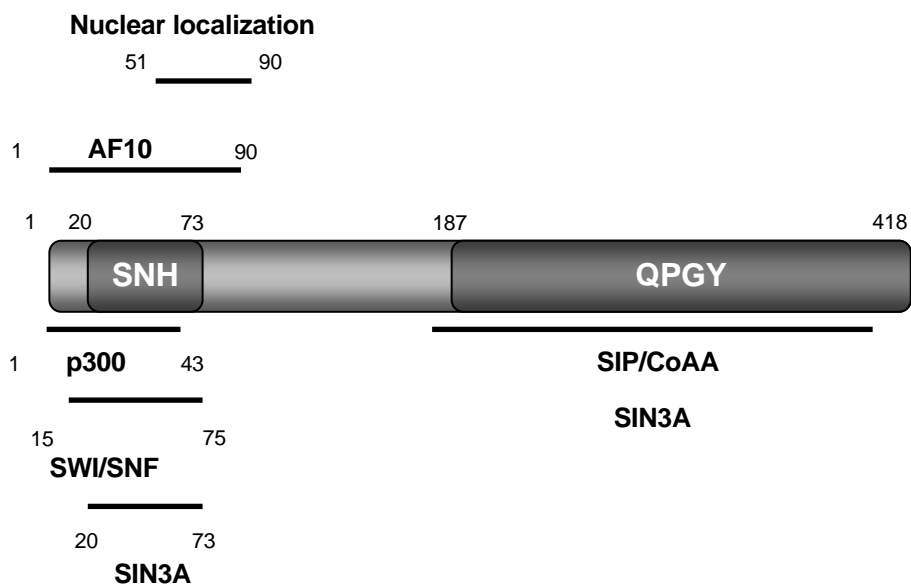*Figure 2. Domain structure of SS18 showing sites of protein-protein interaction*

In addition to it N and C terminal domains, SS18 contains numerous potential sites for protein-protein interaction. A scan of the ELM database (http://elm.eu.org/) for potential interaction motifs reveals at least 36 sites which can serves as potential interaction sites including 13 putative SH2 sites and 15 putative SH3 sites.

The *SS18* gene family

Based on sequence similarity two SS18 paralogs have been identified, all of which encode proteins with conserved SNH domains [34, 38]. Located on chromosome 20 the *SS18L1* gene encodes a 396 amino acid protein with 54% sequence homology with SS18. In contrast to SS18, the SS18L1 displays a more restricted expression profile with expression largely confined to the brain and neuronal tissues. In light of its expression pattern, SS18L1 was shown to be a calcium responsive transcription factor necessary for neuronal and dendritic cell development [39]. The domain structure of SS18L1 is similar to that of SS18, with both proteins containing a QPGY C-terminal region and an identical SNH domain [34]. Interaction wise SS18L1 forms complexes with the histone acetyl transferases, p300 at the N terminal domain and with CBP at the C terminal domain.

Although p300-SS18 interaction has been described, no interaction between CBP and SS18 has been reported. It is of interest to note that the amino acid sequence of the CBP interaction domain in SS18L1 is conserved in SS18 and is localised in the C terminal region which is lost in synovial sarcoma. *SS18L1* has also been implicated in synovial sarcoma as a result of a translocation between chromosome 20 and the X chromosome leading to the expression of a SS18L1-SSX1 fusion protein implying that both SS18 and SS18L1 deregulation may produce similar oncogenic effects. In comparison to its bigger brothers the *SS18L2* gene encodes an 88 amino acid protein consisting solely of a single SNH domain. Since SS18L2 lacks the QPGY transactivation domain present in SS18 and SS18L1 it has been suggested to function as a negative regulator by competing for interaction in target proteins [34].

SS18 expression

The promoter region of *SS18* and its two homologues are TATA-less and embedded in canonical CpG islands, a common characteristic of housekeeping genes [34]. Consistent with this notion RNA in situ hybridization of the mouse homolog, *Ss18* has shown a ubiquitous expression pattern in adult mouse tissues [38]. Analysis of Ss18 expression has identified two alternative splice variants which differ in the presence of a 93 bp exon in the QPGY domain. The 31 amino acid sequence coded for by the unspliced SS18 variant does not contain any putative DNA binding domains; however the sequence is rich in tyrosine, suggests that this domain may be involved in phosphorylation regulation or determining protein conformation. The

larger splice variant Ss18-α displayed mainly embryonic expression whereas the smaller splice variant SS18-β was found to be the main variant expressed in adult tissue. These two variants were also found to be expressed in human tissues and cell lines [20, 40]. Differences in biological properties have been reported with the full length SS18 isoform having greater transcriptional activation, cell proliferation and cell survival properties [41]. The expression of alternative splice variants may also have functional implications for synovial sarcoma pathogenesis. Over expression of the spliced isoform of SS18-SSX was also found to be significantly associated with cyclin A and cyclin E over expression in synovial sarcoma tumors (unpublished results).

SS18 and development: Lessons from mouse models

Studies of *Ss18* knockout mice have shown an absolute requirement for Ss18 during development [42]. *Ss18* knockouts showed significant defects in growth and gestation with embryonic lethality occurring before E9.5. Intriguingly *Ss18* knockouts displayed phenotypical characteristics with those of *p300* knockout mice implying possible co dependence between Ss18 and p300 in development. The lethal affects of Ss18 knockout were due to defects in placental implantation and blood vessel formation. Several target genes were shown to be down regulated in *Ss18* knock out mice including *Pparb1* a nuclear co activator required for micro vessel maturation and angiogenesis. In addition to its role in development *Pparb1* is a p53 interacting protein which can modulate the activation of p53 responsive genes promoting activation of *BAX* and inhibition of *p21*. *Pparb1* expression can also enhance p53 degradation in an ubiquitin-proteasome dependent manner through up regulation of *HDM2* [43-45].

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SS18 protein-protein interactions

Since SS18 lacks any consensus DNA binding motif it presumably performs its functions as a transcriptional activator via protein-protein interactions. Recently several SS18 interacting partners have been identified. The majority of these interactions map to the sequences within or surrounding the conserved SNH domain, suggesting that this domain serves as a molecular platform for protein-protein interactions. The fact that many of these proteins have overlapping binding sequences suggests competition between co-activators for binding to SS18.

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A common feature of many of these interacting partners is that they have been implicated in gene regulation by promoting chromatin modification. The proteins identified include:

p300

SS18 interaction with p300 has been shown to regulate cell adhesion in a transcriptional independent manner [46]. The association of p300 with SS18 is dynamically regulated in respect to adhesion state with complexes forming in mid G1 phase but dissociating before G1-S phase transition, in contact inhibited but not sparsely plated cells. The p300 protein is a large nuclear protein with intrinsic histone acetyl transferase activity which participates in many physiological processes, including proliferation, differentiation and apoptosis [47-49]. p300 can enhance transcription either through functioning as a molecular scaffold and bridging transcription factors to the pol II holoenzyme or by acetylating histones in the vicinity of target promoters thus forcing chromatin into a more open and transcriptional activation permissive conformation [50]. p300 also has numerous non transcriptional functions. Through its association with HDM2, p300 functions as an E4 ubiquitin ligase which can promote the ubiquitination and degradation of p53 [51]. Conversely p300 can also promote p53 stability and transcriptional activity by acetylating key lysine residues in the C terminal of p53 [52].

SWI/SNF

In vitro and *in vivo* SS18 has been shown to interact with BRM1 and BRG1, two mutually exclusive components of the human SWI/SNF protein complex [36, 53, 54]. The SWI/SNF complex is an evolutionary conserved multi-subunit complex involved in the ATP dependent remodelling of chromatin structure which alters the position of nucleosomes at promoter sites of target genes thus making them more accessible to the transcription machinery [55-57]. SWI/SNF activity has been widely associated with transcriptional activation of genes, however the role of SWI/SNF as a transcriptional activator is controversial as increasing evidence suggests that these complexes are also associated with transcriptional repression [53, 58-60]. Several studies support a role for this complex in cancer development, as mutation of several subunits has been implicated in a broad range of human malignancies [60-62]. SWI/SNF complexes regulate exit from the G1 and S phase of the cell cycle by actively repressing cyclin E and cyclin A expression, achieved by reversible

associations with Rb and histone de-acetylase complexes [61]. The activity of the BRG and BRM subunits of the SWI/SNF complex are dynamically regulated during the cell cycle. Both proteins are phosphorylated by cyclin-CDK complexes on the G2-M phase transition resulting in the degradation of the BRM1 subunit and the simultaneous nuclear export of BRG. This mechanism of SWI/SNF silencing is presumed to result in the transcriptional arrest necessary for the onset of mitosis [61, 63, 64]. It had been originally proposed that SS18 may recruit the SWI/SNF complex to unwind chromatin and facilitate its own putative trans-activating function, however the discovery that SWI/SNF is also involved in transcriptional repression complicates this somewhat. Both BRG and BRM1 bind to the conserved SNH domain present in the SS18 protein family [36, 53, 54]. Binding of BRM or BRG to the SNH domain actively represses the transcriptional activity of SS18 in a mechanisms requiring the ATPase activity of the BRM1/BRG subunits [53]. It is currently unknown whether this SS18 inhibition is constitutive or dependent on the recruitment of activator/repressor accessories to the SWI/SNF-SS18 complex.

SIN3A

Yeast hybrid analysis identified SIN3A a component of the histone deacetylase complex, as an SS18 interacting partner [65]. SIN3A along with several other subunits form HDAC complexes which act on chromatin to produce a more condensed and transcriptionally inactive state. In contrast to the p300 and SWI/SNF complexes, HDACs primarily function in the repression of gene transcription by removing acetyl groups on histones, recruiting co-repressors and methyl-CpG binding proteins all of which function to maintain the chromatin in a inaccessible conformation [66, 67]. Through mutation analysis two potential SIN3A interaction sites were mapped, one in the conserved SNH domain and a second in the QPGY region. A negative SIN3A regulatory domain localised in the last 8 terminal amino acids was also proposed as deletion of this region effectively enhanced SIN3A association with SS18. The interaction of SIN3A with SS18 could abrogate the transcriptional activity mediated by SS18 suggesting SIN3A is a negative regulator of SS18 [65].

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AF10

Through two hybrid screening SS18 was shown to interact with AF10, a member of the zinc finger/leucine zipper family of transcription factors [35]. Although AF10 downstream targets have yet to be identified, its expression is deregulated in t(10;11) positive acute leukaemia's when it is fused in frame with MLL [68]. SS18 interaction with AF10 may form a bipartite co-activator complex and be targeted to specific gene promoters through the DNA binding domain present in AF10 [35].

SIP/CoAA

SS18 interacting protein/co-activator activator is a nuclear co-activator and RNA splicing modulator protein containing a motif similar to those found in the EWS and in TLS/FUS family of transcriptional activators [69]. In comparison with other SS18 interacting partners which interact with domains clustered within or around the SNH domain, the SIP/CoAA interaction domain was found localized in the QPGY domain in both SS18 and SS18-SSX. SS18 acts together with SIP/CoAA in stimulating estrogen and glucocorticoid receptor-dependent transcriptional activation in a hormone-dependent manner. This mechanism was dependent on the presence of a functional hBRM and/or BRG1 and an intact SNH domain.

SSX: The trans repressor

The *SSX* genes constitute a highly conserved family of at least nine members localized on chromosome band Xp11.2 [10, 11]. Unlike SS18, the expression of SSX is severely restricted, expressed only in testis, thyroid and in a variety of human tumours [70, 71]. Dormant *SSX* genes can be activated when cultured cells are treated with a demethylating agent 5-aza-deoxycytidine or the histone deacetylase inhibitor trichostatin A, suggesting that the expression of the *SSX* genes is ultimately controlled by epigenetic regulation [24]. Based on these observations and by the fact that SSX can illicit an immune response, SSX is classified as a cancer testis antigen.

All *SSX* genes consist of seven exons and encode proteins of 188 amino acids [23]. *In vitro* studies have shown that the SSX proteins have the ability to repress the transcription of reporter genes by fifty fold when expressed in NIH3T3 fibroblasts [36, 72]. The homology between the SSX family members is high ranging from 88% to 95% at the protein level.

Divergence between family members occurs in a divergent domain located in the C terminal portion of the protein [72]. The SSX proteins are rich in charged amino acids and contain an acidic C terminal tail. Sequence analysis and functional studies have identified two major domains in SSX.

The N terminal end of the SSX protein exhibits extensive homology to the Kruppel-associated box (KRAB), a domain present in a sub group of zinc finger, transcriptional repressor proteins [73]. However, the SSX KRAB-like domain appears to be an inefficient or even inactive repressor domain [36, 72].
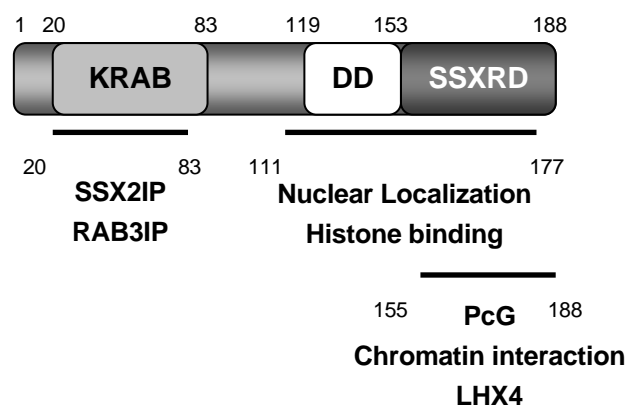

Figure 3. Domain structure of SSX showing sites of protein-protein interaction

The majority of repressor activity of the SSX proteins has been mapped to a novel 34 aa C terminal domain termed the SSXRD. The C terminal SSXRD is the most highly conserved portion of the SSX protein possibly indicating its critical function in SSX mediated gene regulation [37].

The *SSX* gene family

In addition to the 9 protein coding genes, 10 pseudo genes have also been identified [74]. All pseudo genes with the exception of one on chromosome 6, are located on the X chromosome. The *SSX* genes are found clustered within two loci about 300kb apart. *SSX1* is clustered with *SSX3*, *4, 5, 6, 9,* and 6 *SSX* pseudogenes at Xp11.23-p11.3, while *SSX2, 7, 8,* and three other pseudo genes cluster at Xp11.4. This suggests that the two *SSX* gene clusters arose from a duplication encompassing a region of approximately 100kb. The gene duplication events that resulted in the generation of the SSX gene family are still ongoing. Analysis of a genomic clone (GeneBank #AL450023) shows a

recent, almost exact, duplication of a 70kb segment that includes *SSX2* leading to two copies of *SSX2* with identical coding sequences in the genome [75].

SSX protein-protein interactions

The SSX proteins are localized in the nucleus, where they display diffuse nuclear staining [37]. Deletional studies have demonstrated that the SSXRD domain is responsible for SSX nuclear localization, however similar to SS18, no consensus DNA binding domain has yet been identified, implying that SSX requires an interaction with one or more DNA binding proteins. To date several interacting partners for SSX have been identified:

PcG Bodies and core histones

Immunofluorescence studies have shown that the SSX proteins co-localize with the PcG complex of proteins in nuclear speckles [76]. Various PcG proteins have been found to associate with SSX including HPC2, BMI1 and RING1 [72]. The PcG proteins are thought to maintain the repressed state of target genes throughout cell divisions by inducing changes in chromatin structure thus inhibiting access of the transcriptional machinery to target genes. However in spite of the co localization no direct physical interaction between recombinant SSX and any of the PcG proteins has been observed in vitro [72]. The possibility remains that an as yet unidentified associating protein mediates the association between SSX and PcG proteins via the SSXRD. The C-terminal region of SSX also binds strongly to core histones and oligonucleosomes *in vitro*. Consistent with this SSX has been shown to interact with mitotic chromosomes and remain nuclear localized even during the later stages of cell division [37, 54].

SSX2IP

SSX2IP is a 614 amino acid protein which contains several domains and a putative NLS. SSX2IP was initially identified as an interacting partner with SSX2 and SSX3 but not with the other SSX family members [77]. The SSXIP interaction domain was found to be localized in the N terminus of the SSX protein. SSX2IP was also identified as a leukaemia associated antigen expressed in AML patients [78]. The murine homologue of SSX2IP known as ADIP has been shown to interact with afadin

(AF-6 in humans) which is thought to play a role in signal transduction at special cell–cell junctions [79].

RAB3IP

Using a yeast two-hybrid system RAB3IP was identified as an SSX2 specific binding partner [77]. RAB3IP is a ubiquitously expressed cytoplasmic protein. Six isoforms have been identified all of which contain an ER retention signal. Similar to SSX2IP the RAB3IP interaction domain in SSX2 was mapped to the N terminal KRAB domain. Immunofluorescence studies shown that the RAB3IP protein is normally localized in the cytoplasm however co expression with SSX2 could promote relocalization to the nucleus.

LHX4

A yeast two-hybrid screen identified the transcriptional activatorLHX4 as an interaction partner for SSX1 [80]. LHX4 is a LIM homeobox DNA binding protein involved in development [81, 82]. Through functional mapping LHX4 was found to interact with the C terminal RD domain of SSX. LHX over expression has been observed in several haematological malignancies where its expression is linked to proliferation and cell survival [83]. Interaction of SSX with LHX4 is presumed to repress the transcription of target promoters containing LHX4 binding sites, through the ability of SSX to recruit components of the PcG complexes involved in transcriptional silencing.

SSX expression in cancer

The expression of the *SSX* genes is highly variable between different tumor types. RT-PCR analysis of the expression of five *SSX* genes in 325 specimens of human tumors of various types showed *SSX1*, *SSX2*, and *SSX4* were expressed in 8%, 15%, and 15% of all tumors, respectively, with a low frequency of *SSX5* expression and only one case of *SSX3* expression [71]. At least one *SSX* family member was expressed in 57% of head and neck cancers, in 50% of ovarian cancers, in 43% of melanomas, in 27% of colorectal cancers, and in 23% of breast cancers, whereas leukemias, leiomyosarcomas, seminomas, and thyroid cancers did not express any detectable SSX. The correlation between SSX expression and tumor progression is unclear although several reports suggest that SSX expression is linked with poor clinical outcome and tumor development. Analysis of SSX mRNA expression in 211 bone and soft tissue tumors showed significantly higher expression of SSX in malignant tumors when compared

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with benign lesions. A correlation between clinical grade and *SSX* mRNA expression was also reported with SSX expression levels higher in stage III tumors than that in stage I or II tumors [84, 85]. Studies on myeloma patients has effectively demonstrated that groups which expressed 5 *SSX* genes, *SSX1-5* had a clinically significant reduced survival time when compared with those patients expressing 3 or fewer *SSX* genes. The *SSX* gene expressed could also have clinical consequences with the expression of *SSX1*, *SSX2* and *SSX5*, but not *SSX4* associated with reduced survival. In addition the spectrum of *SSX* genes expressed also clinical consequences with SSX2 expressing tumors displaying the strongest correlation with reduced survival [86].

SSX as a therapeutic target

Based on its immunogenicity and restricted expression, cancer vaccines targeting SSX have been speculated. The presence of anti SSX antibodies in the circulation of melanoma patients has raised the possibility that SSX proteins may serve as tempting targets for future immunotherapeutic strategies [71, 87]. SSX expression has been shown to elicit a cytotoxic T-cell response as demonstrated by the generation of SSX specific CTLs *in vitro* and in breast cancer patients [88, 89].

SS18-SSX: A transcription factor with multiple personalities.

Analysis of the domain structure of SS18 and SSX suggests that both proteins function as epigenetic regulators through interaction with site specific DNA binding proteins and with chromatin remodelling complexes. In the majority of SS18-SSX fusions, the last 8 amino acids of SS18 are lost and replaced by the last 78 amino acids of SSX1, SSX2 or SSX4 [24]. The typical SS18-SSX fusion protein includes several domains from both SS18 and SSX. As such SS18-SSX may be a class of ''activator-repressors'' which have dual functions in transcriptional regulation [90].

The trans-activating domain of SS18 are virtually all retained in the fusion protein however it seems that the trans-activating ability of SS18 is reduced due to the attachment of the SSX portion containing the SSXRD [33, 72]. The presence of SNH domain from SS18 and the SSXRD domain of SSX implies that these proteins can potentially associate with functionally antagonizing complexes, p300 and SWI/SNF complexes, involved in trans-activation and the PcG protein complexes involved in repression [36, 76, 91].
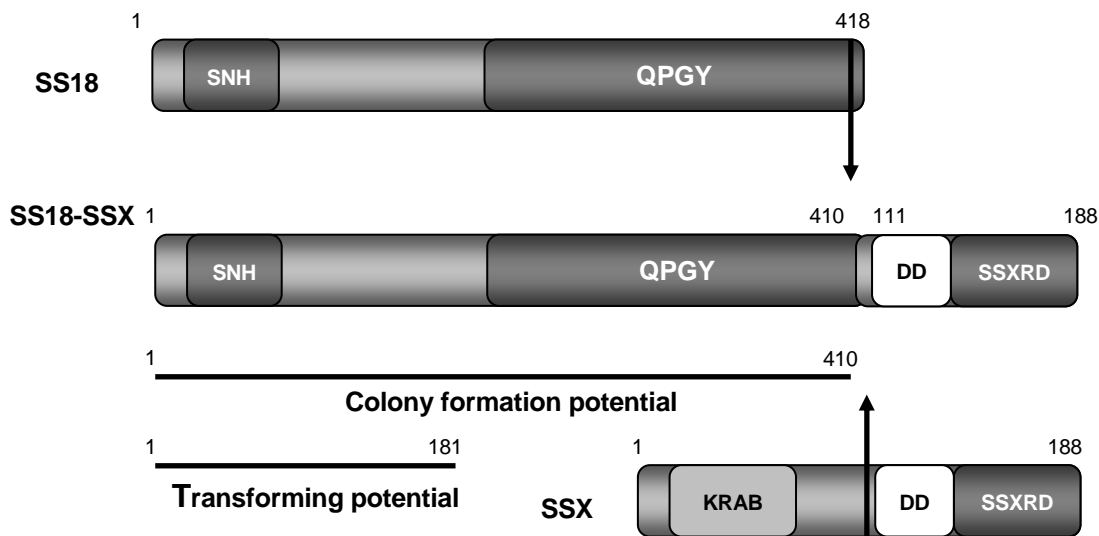
F*igure 4. Schematic overview of the structural and functional domains
of the SS18-SSX protein.*

SS18-SSX: Loss of function vs. gain of function?

Analysis of the domain structures of SS18 and SSX suggest both potential loss of
normal function and the gain of function as potential oncogenic mechanisms of SS18-
SSX. Several finding suggest the transforming potential of SS18-SSX comes from the
SS18 domain. Over expression of SS18-SSX enhances growth rate in culture,
anchorage-independent growth and tumor-forming potential in nude mice [92].
Experiments with deletional mutants of SS18-SSX have mapped the transforming
potential of the SS18-SSX fusion to the N-terminal 181 amino acids of SS18. However,
wild type SS18 lacked transforming activity indicating that the additional sequences
derived from SSX or loss of the C terminal of SS18 are required for SS18-SSX
transforming activity [92].

The importance of an intact SS18 C terminal domain was also highlighted in mediating
SS18-p300 function [46]. Studies using deletion mutants have shown that the loss of
the last 8 amino acids in the C terminal region of SS18 results in a 79% decrease in cell
adhesion to a fibronectin matrix, down regulation of β-integrin activity and loss of cell
contact inhibition. Deletion of this sequence did not abolish p300 binding implying
possible defects in p300-SS18 mediated signaling. In synovial sarcoma the fusion of

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SSX results in a loss of these amino acids necessary for p300-SS18 cell adhesion function which may have implications in the ability of these tumors to metastasize.

Experiments using promoter activation studies have shown an unexpected property of SS18-SSX that as an activator of the cell cycle regulator p21. Exogenous over expression of SS18-SSX effectively enhanced the activity of a p21 promoter construct in a p53 independent manner [93] (unpublished observations). This effect was specific for SS18-SSX since neither wild type SS18 or SSX had the same transcriptional activation effects. However, a C terminal deletion mutant of SS18 lacking the C terminal domain was just as efficient as SS18-SSX in inducing p21 transcription. Collectively these experiments suggest that the majority of the transforming potential of the SS18-SSX fusion protein lies within the SS18 portion and that loss of the C terminal portion of SS18 is sufficient to induce certain regulatory changes similar to those induced by SS18-SSX expression.

A dominant negative property was suggested based on the ability of SS18 to oligomerize via the QPGY domain [94]. Co expression of SS18 with SS18-SSX resulted in the formation of hetero-oligomers which was proposed to inhibit the transactivation potential of wild type SS18, however a direct effect of SS18/SS18-SSX hetero oligomerization on trans activation was not established [95].

An indication that the SSX domain may not be essential *per se* is the recent characterization of several variants of SS18-SSX. In all cases the SS18 portion of the fusion is maintained but differences in the translocation breakpoint result in variability in the SSX portion [24]. A single case of an SS18-SSX4 expressing synovial sarcoma was described where the resulting gene fusion resulted in a downstream shift in the open reading frame of the SSX4 portion, resulting in the translation of a novel fusion protein which lacked any recognizable SSX domains [20]. A recently established cell line was also characterized which expressed an SS18-SSX1 fusion protein with a C terminal truncation. As a result this fusion protein lacks the entire SSXRD. Unlike other synovial sarcoma cell lines, it grows slowly *in vitro* and is non-tumorigenic in nude mice. This may indicate that SSX is dispensable for tumorgenesis but may an important contributor to tumor aggressiveness [96].

SS18-SSX: Rewiring the cell cycle

In light of the transforming potential of SS18-SSX several studies have focused on the expression of SS18-SSX with that of cell cycle regulators. One study reported the over expression of cyclin D1 in around 60% of synovial sarcomas which coincided with nuclear expression of cyclin E in about 30% of the cases [97]. Analysis of samples of localized synovial sarcoma reported a link between high levels of cyclin A and cyclin D1 (but not cyclin E) and the expression of SS18-SSX1, suggesting that the more aggressive phenotype of SS18-SSX1 may be as a consequence of accelerated tumor proliferation [98]. In addition analysis of alternative splicing of SS18-SSX has also shown a correlation with the expression of the spliced isoform of SS18-SSX and increased expression of cyclin E and cyclin A (unpublished results). A recent report provided evidence that high levels of cyclin D1 expression found in synovial sarcomas may be due to increased stability of the cyclin D1 protein [99]. SS18-SSX expression was found to be necessary to stabilize cyclin D1 levels in a manner independent of the canonical GSK3β ubiquitin proteasome pathway. A second report also showed that SS18-SSX expression could directly activate the expression of the *cyclin D1* gene [90]. In addition to promoting the expression of positive regulators of cell cycle progression, SS18-SSX expression has also been implicated in inducing the expression of the cyclin-CDK inhibitor p21 [93, 100]. A second cell cycle regulator COM1 was also reported to be effected by SS18-SSX expression. Micro array analysis of SS18-SSX1 expressing HeLa cells showed that SS18-SSX1 directly down-regulated the expression of COM1, a regulator of cell proliferation. COM1 was found to be expressed at relatively low levels in synovial sarcoma tissues and cell lines. In addition conditional over expression of COM1 induced apoptosis and reduced cell growth implying that SS18-SSX1 can disable COM1 tumor suppressive function by down regulating its expression [95].

SS18-SSX: The E cadherin, Wnt and ephrin pathways.

Historically synovial sarcomas were classed based on morphological appearance where tumors appeared as monophasic, composed of spindle cells or biphasic composed of spindle and epithelial cells. Typically SS18-SSX2 expressing tumors tend to be monophasic whereas SS18-SSX1 tumors were biphasic; however this consensus has

been challenged [28]. A suggested mechanism for this association was the differential regulation of E cadherin expression by SS18-SSX1 and SS18-SSX2 [101].

SS18-SSX1 and SS18-SSX2 were shown to preferentially disable the Snail or Slug mediated repression of E cadherin transcription respectively; suggesting a mechanism by which SS18-SSX can promote E cadherin mediated epithelial phenotypes. Since Snail is a more potent repressor of E cadherin expression than Slug, the ability of SS18-SSX1 to preferentially inhibit Snail mediated trans repression of the E cadherin promoter may explain why SS18-SSX1 tumors show higher levels of E cadherin expression and more pronounced epithelial characteristics. Several groups have reported the involvement of the Wnt signaling pathway in the morphogenesis and/or tumorgenesis of synovial sarcoma. Evaluation of E-cadherin, expression and associated proteins (α, β, λ, -catenins and p120) in a cohort of synovial sarcomas showed immunoreactivity for E-cadherin, α-catenin, β-catenin, and p120 in all specimens. Tumor samples displayed high levels of nuclear and cytoplasmic staining for β catenin, suggesting constitutional activation of the Wnt pathway in synovial sarcoma [102]. Subsequent nuclear localization of β catenin in was shown to be dependent on the ability of SS18-SSX2 to recruit and retain β catenin in the nucleus. However is spite of the high levels of nuclear sequestered β catenin induced by SS18-SSX the expression of known target genes of canonical Wnt signaling were not up regulated [103]. Experiments using an inducible system of SS18-SSX expression did however show induction of certain components of the Wnt signaling network. In this set of experiments cyclin D1 and CXX5, a potential inhibitor of Wnt signaling was up regulated following expression of SS18-SSX2 [90].

SS18-SSX expression has been implicated in cytoskeleton remodeling, stabilization of the microtubule network and resistance to microtubule inhibitors. A recent report has suggested that expression of SS18-SSX can alter cell phenotype, promoting morphological changes consistent with differentiation [104]. Activation of the ephrin signaling pathway was implicated in the induction of phenotypical changes consistent with neuron outgrowth and cell repulsion. The activation of EphB2 signaling was a property of the SS18-SSX2 fusion protein since neither SS18 nor a deletional SS18 construct lacking the last 8 aa could induce changes in cell morphology. Consistent with this EphB2 over expression was detected in SS18-SSX2 expressing synovial sarcoma tumors.

SS18-SSX and epigenetic regulation

Recent evidence suggests that both wild type SS18 and SSX and the SS18-SSX fusion protein function as epigenetic co regulators of transcription. The interaction of SS18 with DNA binding factors such as AF10 would facilitate the recruitment of chromatin re-modelers such as p300 or components of the SWI/SNF complex thus promoting changes in chromatin structure and the activation of target genes. Conversely the association of SSX with DNA binding proteins such as LHX4 could facilitate the transcriptional repression through localization of PcG proteins at the promoter sites of target genes. In the fusion of SS18 with SSX the resultant fusion protein retains the ability to interact with both positive (p300 and SWI/SNF) and negative (PcG) regulators of chromatin structure and with DNA binding transcription factors (AF10 and LHX4). It is therefore highly likely that the SS18-SSX fusion protein uses these domains acquired from both parent proteins to promote aberrant epigenetic alteration of target genes. Indeed studies of the IGF axis suggest that SS18-SSX expression can deregulate components of the IGF signaling network. Over expression of IGF2 has been shown in synovial sarcoma where its expression is required to maintain SS18-SSX induced oncogenicicity [105, 106]. Recently the mechanism of IGF2 over expression was shown to be as a consequence of SS18-SSX induced epigenetic deregulation of the IGF2 promoter [90]. Over expression of SS18-SSX2 effectively induced enhanced acetylation and methylation of specific histones in the vicinity of the *IGF2* promoter. Increased association of the BRM component of the SWI/SNF complex with the *IGF2* and *CD44* promoter as a result of the expression of SS18-SSX2 was also reported, suggesting that SS18-SSX can interfere with SWI/SNF mediated epigenetic regulation.

Treatment strategies for synovial sarcoma

Synovial sarcoma is generally regarded as a high-grade sarcoma. Treatment regimes usually involve surgery to remove tumor tissue and adjunctive radiotherapy; however remission is extremely common and approximately half of the patients with localized tumor at diagnosis eventually develop metastases [107]. Previous clinical studies have shown that treatment with doxorubicin in conjunction with ifosfamide may be of therapeutic benefit in treatment strategies for synovial sarcoma, however the adverse side effects and the ''hit and miss'' nature of these treatments suggest a need to develop

therapies which target the molecular pathways deregulated by SS18-SSX expression [108, 109]. The generation of vaccines using epitopes spanning the fusion breakpoint have been suggested [110, 111]. Treatment of six synovial sarcoma patients with a 9 mer peptide spanning the SS18-SSX breakpoint induced peptide specific CTLs in four patients and tumor remission in one [110]. Several recent reports have suggested the use of non conventional drugs as potential treatment regimes. Using 3 dimensional spheroid *in vitro* models of synovial sarcoma *Terry et al* assessed the growth inhibitory properties of a panel of conventional RTK inhibitors gefitinib, NVP-AEW541, imatinib mesylate (Gleevec), SU5402, PRO-001, trastuzumab (Herceptin), and the HSP90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG) [112]. The majority of the RTK inhibitors inhibited cell proliferation only at high concentrations which were not clinically applicable. In contrast 17-AAG was a potent inhibitor of cell growth and rapidly induced apoptosis in synovial sarcoma cells which the authors attributed to the ability of 17-AAG to inhibit protein folding of nascent RTKs. A second potent inhibitor of synovial cell growth was also identified [113]. FK228 is an inhibitor of the histone deacetylase complex. Treatment of a panel of cells showed that FK228 significantly suppressed the growth of synovial sarcoma cells when compared with other types of soft tissue tumor. Further more exogenous expression of SS18-SSX in a non tumorigenic cell line enhanced the sensitivity of these cells to FK228 treatment. In vivo FK228 inhibited the growth and metastases of synovial sarcoma cells. Considering the recent data which implicate SS18-SSX in inducing epigenetic deregulation of target genes, it is tempting to suggest that FK228 may counteract the effect of SS18-SSX in altering the activation of target genes [90].

The p53 tumor suppressor: A universal target for cancer progression.

The *p53* gene encodes one of the most intensively studied tumor suppressor proteins within the molecular cancer field. Its biological relevance as "guardian of the genome" is supported by the fact that more than 50 percent of all cancers carry inactivating point mutations in the DNA binding domain, resulting in abrogated transactivation function [114, 115]. In the absence of p53 mutation tumor cells may acquire the ability to circumvent the p53 circuit by altering upstream or down stream signaling cascades which effect p53 stability, sub cellular localization or trans activating potential.

Mechanisms of p53 mediated tumor suppression.

The p53 tumor suppressor plays a pivotal role in mediating the cellular response to a wide range of environmental and intra-cellular stresses including DNA damage, oncogene activation, ribosomal stress, hypoxia and contact inhibition [116]. Although the protein is present at very low levels under basal conditions it is rapidly stabilized in response to stress allowing for accumulation and activation of p53 function. The tumor suppressive function of p53 is primarily due to its ability to act as a transcription factor that regulates the expression of a large number of target genes [117]. Through this mechanism p53 can induce a number of different responses, ranging from the induction of cell cycle arrest genes, apoptosis, DNA repair and senescence [116]. In addition to its role as a transcriptional activator, p53 has also been shown to have transcriptional independent activities stemming from its ability to induce apoptosis by catalyzing the Bax/Bak mediated permeabilization of the mitochondrial membrane [118-120].

Regulating p53 function via HDM2

p53 levels are tightly regulated through interaction with its negative regulator HDM2. The murine equivalent of the *HDM2* gene (*MDM2*) was initially identified from double minute chromosome fragments isolated from spontaneously transformed murine 3T3 cells [121]. Subsequent molecular studies have shown that the *HDM2* gene is over expressed in numerous cancers, particularly sarcomas underscoring its pivotal involvement in the development of malignancies [122, 123]. The *HDM2* gene encodes a 491 aa nuclear phospho protein with several conserved domains [124, 125]. The *HDM2* gene is itself a target for p53 and the two proteins therefore function within an auto-regulatory loop in which HDM2 can keep the physiological levels of p53 low and contribute to the recovery phase at the end of a p53 response [126]. HDM2 can regulate p53 by several mechanisms:

HDM2 promotes p53 ubiquitination and degradation.
The C terminus of HDM2 contains a RING finger domain which is required for HDM2 ubiquitin E3 ligase activity [127]. The principle mechanism of HDM2 mediated p53 regulation is due to its intrinsic ubiquitin ligase activity which catalyses the addition of ubiquitin moieties to lysine residues in the C terminus of p53 [128-131]. The addition of ubiquitin groups act as a ''destabilizing'' signal which promotes p53 degradation via

cytoplasmic or nuclear proteasome complexes. HDM2 mediated p53 ubiquitination takes place in a ternary complex with the p300 co activator protein.

Studies using HDM2 mutants deficient for p300 binding showed that HDM2 alone could effectively catalyze p53 mono ubiquitination, however this was inefficient for protein degradation [131, 132]. Proteasome targeting and degradation of p53 requires both the E3 type ligase of HDM2 and the E4 type poly ubiquitination activity of p300 [133, 134]. Experiments with knock out mice revealed that deletion of the *HDM2* gene is embryonic lethal but are rescued in a *p53* null background, implying that HDM2 is the principle ubiquitin ligase regulating p53 function during development [124, 125, 135].

HDM2 abrogates p53 mediated trans activation.

In addition to mediating p53 ubiquitination, HDM2 can also regulate p53 via alternative mechanisms. The binding of HDM2 to the N terminal domain of p53 can abrogate p53 mediated transactivation by occluding sites of interaction with the transcriptional machinery [123, 136]. A perhaps more indirect mechanism for regulating p53 function also exists whereby HDM2 bound to p53 can ubiquitnate histones associated with p53 target genes thus promoting the formation of inactive chromatin [137].

HDM2 promotes nuclear to cytoplasmic shuttling

HDM2 can alter p53 sub cellular localization promoting the shuttling of p53 from its site of action in the nucleus to the cytoplasm in a ubiquitin ligase dependent manner [51, 138, 139]. Although it was initially proposed that HDM2 acted as a molecular chaperone in the export of p53, it is now known that the HDM2-p53 complex does not exit the nucleus in association. Instead the mono-ubiquitination of the C terminal of p53 induced by HDM2 leads to a conformational change in p53 causing exposure of nuclear export signals required for efficient translocation [51, 140]. The traditional view was that nuclear to cytoplasmic transport of p53 was a pre-requisite for p53 degradation by cytoplasmic proteasome complexes [141, 142].

However recent results suggest that nuclear proteasomes are probably the primary site for degrading ubiquitinated p53, whereas cytoplasmic shuttling of ubiquitinated p53 may augment p53 signaling or down regulate the p53 nuclear response following a stress signal [143].

Stress signals break the HDM2-p53 circuit

The p53-HDM2 loop is a convergence point for numerous types of cellular stress which activate p53. Oncogene activation inhibits HDM2 function via the activation of Arf [144]. DNA damage and other stresses reduce the ability of HDM2 to interact with p53 leading to p53 stabilization and transactivation. Traditionally HDM2 was believed to bind p53 under normal conditions and promote its turnover whereas, upon stress p53 and HDM2 become increasing phosphorylated at specific residues which abrogate HDM2-p53 interaction [145]. However this view has become challenged following conflicting *in vitro* results and studies of mutant p53 mouse models where N terminal phosphorylation site mutants of p53 were still effectively stabilized [146, 147]. At the moment the data suggests that N terminal phosphorylation of p53 may partially inhibit HDM2-p53 interaction but full inhibition requires additional mechanisms. An emerging concept is the importance of HDM2 levels in regulating the p53 response. HDM2 possesses the activity of an E3 ubiquitin ligase which mediates its own autoubiquitination as well as the ubiquitination of other substrates. The balance between auto- and substrate-ubiquitination of HDM2 is modulated physiologically by posttranslational modifications [148]. Studies have reported that destabilization of HDM2 is an important factor for initiating a p53 response [149]. Expression of TAFII250 can abrogate p53 function by inhibiting HDM2 autoubiquitination [150]. Activation of RTKs can promote p53 degradation via promoting stabilizing phosphorylation of HDM2 within the central domain [151]. More recently a single nucleotide polymorphism was found in the HDM2 promoter which resulted in enhanced transcription which was sufficient to inhibit p53 function [152, 153]. The fact that small molecule inhibitors that inhibit HDM2 binding are sufficient to activate p53 suggests that the most critical requirement for p53 activation is abrogation of HDM2 binding.

Targeting the p53-HDM2 axis.

HDM2 has emerged as a potential target for re activating tumors with wild type p53. Recently the first potent and selective small-molecule antagonists of the p53-HDM2 interaction, the nutlins, were identified [154]. Nutlins abrogate HDM2 function by binding to the hydrophobic p53 binding pocket on the surface of HDM2 thus preventing p53-HDM2 interaction. The anti-tumor effect was observed in cells carrying wild type p53 but not in cells with mutant or deleted p53 suggesting that the anti tumor

effect of nutlin is dependent on a functional p53 pathway. *In vivo* nutlin treatment was well tolerated in osteosarcoma xenographs leading to a 90% inhibition of tumor growth [154]. Nutlin has recently been shown to be effective in inducing a p53 in a variety of cancer cells including liposarcomas osteosarcomas neuroblastoma retinoblastoma and several classes of leukemia [155-162].

Aims of Thesis

The general aim of the thesis was to characterize the functions of the SS18 and SSX transcription factors and their respective role in tumorgenesis.

The specific aims of these studies were;

- **Paper I:** To investigate the expression profile of several classes of CT-antigens in stem cells. More specifically to study the potential role of the synovial sarcoma associated CT-antigen SSX in maintaining a stem cell like state.

- **Paper II:** To investigate the potential oncogenic function of SSX expression in promoting cell growth and cell cycle progression *in vitro* and *in vivo* by utilizing a siRNA knockdown strategy.

- **Paper III:** To study the effect of SS18-SSX expression on the function of p53 and to determine the mechanism by which synovial sarcoma cells can evade p53 mediated tumor suppression.

- **Paper IV:** To determine the effect and possible therapeutic potential of inhibitors of the p53-HDM2 auto regulatory loop in restoring p53 function in synovial sarcoma cells.

Results & Discussion

Paper I

Cancer/testis antigen expression in human mesenchymal stem cells: down-regulation of SSX impairs cell migration and matrix metalloproteinase 2 expression.

The cancer testis antigens are a diverse class of genes usually localized on the X chromosome which under normal conditions are only expressed in germ cells and placenta. Aberrant expression of CT genes is also frequently observed in cancer cells possibly due to a loss of epigenetic regulation. Although over 40 different families of CT antigens have been identified their functional role in development and cancer is poorly understood.

In paper I we show that in addition to the traditional sites of CT antigen expression, germ and cancer cells; CT antigens are also expressed in undifferentiated mesenchymal stem cells. Several CT genes including *SSX, NY-ESO-1*, and *N-RAGE,* were expressed in undifferentiated mesenchymal stem cells (MSCs) and down-regulated after osteocyte and adipocyte differentiation.

To elucidate the possible overlapping function played by these genes in cancer and stem cells, a comparative analysis of the localization of SSX was made. The cellular localization of SSX was made in relation to other mesenchymal proteins including cell adhesion (fibronectin and laminin), matrix (vimentin), or metallo-proteinase (MMP2) molecules. SSX was located in the cytoplasm of MSCs, exhibiting a finely granulated pattern. Analysis of co localization revealed overlap between SSX and MMP2 and SSX and vimentin, however immunoprecipitation experiments failed to detect a direct interaction.

Vimentin and MMP2 are mesenchymal proteins whose expression is also associated with tissue invasion in several tumor types. In light of the relationship between SSX and these proteins we analyzed the migratory capacity of a highly metastatic melanoma cell line, DFW which expresses high levels of SSX, using a conditional SSX RNAi system. We found that knockdown of SSX expression was associated with a decrease in MMP2 expression and associated with impaired migration

These results support a functional role for SSX in normal stem cell migration and suggest a potentially role for SSX in promoting a mesenchymal phenotype and in cancer cell metastases.

Paper II

SSX activates β-catenin transcriptional function and sustains tumor cell proliferation in vitro and in vivo.

The *SSX* genes comprise a highly conserved family whose abnormal expression is observed in a variety of human tumors. In paper II we further define a role for SSX in tumorgenesis through the generation and characterization of an inducible RNAi system capable of knocking down SSX expression.

We designed a degenerate shRNA cassette specific for the 3'utr of SSX1-5 which was cloned into the pSuper and pSuperior siRNA expression vectors. For inducible siRNA expression the pSuperior vector was transfected into the melanoma cell line DFW which expresses *SSX1-5*, under the control of a tetracycline repressor. Addition of doxycycline to the cell culture medium resulted in a rapid down regulation of SSX with minimal protein levels detected after 24 hrs doxycycline addition. To determine the effect of SSX expression on cell growth and survival we performed viability assays on DFW cells grown in the presence or absence of SSX. We found that SSX knockdown cells displayed significantly slower growth times and clonogenic potential when compared with controls. In order to investigate if the defects in cell proliferation observed after SSX knockdown were due to alteration in cell cycle progression a BrdU incorporation assay was performed. Asynchronous DFW cells were grown in the presence of doxycycline for the indicated time points to induce knockdown of SSX expression. Consistent with the cell proliferation data, knockdown of SSX dramatically decreased the number of cells in the S phase of the cell cycle with cells accumulating in the G1 and G2 compartment of the cell cycle. Our results suggest that SSX knockdown cells have reduced ability to enter S phase. This was further confirmed by analyzing the effect of SSX knockdown on the cell cycle progression using a double thymidine block to obtain cells synchronized at the G_1/S phase boundary. We found that SSX expressing cells rapidly progressed from G1 into S phase upon thymidine removal which was in contrast with SSX knockdown cells which remained trapped at G1/S phase boundary. Consistent with the observed defect in G1 exit, analysis of cyclin expression showed that SSX expressing cells had high levels of cyclin A and cyclin E in comparison to SSX knockdown cells which displayed reduced expression of both cyclins.

DFW melanoma cells were injected subcutaneously into the flanks of immunodeficient SCID mice. For doxycycline regulated expression of siRNA-SSX, a doxycycline or

placebo pellet was introduced into the mice 2 days after initial tumor xenograft transplantation. Macroscopically DFW cells expressing SSX grew significantly faster and induced vessel formation in comparison with SSX knockdown tumors which displayed significant areas of necrosis and contained few proliferative cells. SSX positive tumors were characterized by high proliferation index as determined by the expression KI-67, cyclin D and nuclear accumulation of β catenin. In SSX knocked-down tumors, large necrotic areas were observed, surrounded by proliferating cells with nuclear but mostly cytoplasmic β catenin expression.

This study gives a proof of concept of the oncogenic function of SSX in promoting tumor cell growth.

Paper III

The oncoprotein SS18-SSX1 promotes p53 ubiquitination and degradation by enhancing HDM2 stability.

The deactivation of the p53 tumor suppressor pathway is probably a universal event in the multi-step development of cancer. Although deactivation of p53 function by point mutations occurs in over 50% of cancers, numerous alternative mechanisms exist for disabling p53 in tumors which retain wild type p53. Synovial sarcomas, which express SS18-SSX as a result of a chromosomal translocation rarely display mutation of the p53 gene; implying that defects in upstream or down stream signaling are responsible for loss of p53 mediated tumor suppression. In paper III we investigate the effect of the expression of the synovial sarcoma associated transcription factor SS18-SSX1 on the p53 pathway.

Firstly we showed that exogenous over expression of SS18-SSX decreased p53 protein levels in cell lines which retained wild type p53. In support of this observation methionine pulse chase analysis of p53 levels in U2OS cells showed that p53 half-life was dramatically reduced in the presence of SS18-SSX1. In colony formation assays, co expression of SS18-SSX and p53 could effectively abrogate the anti survival effect of p53. Analysis of p53 levels following the induction of a stress response by either actinomycin D or doxorubicin showed that SS18-SSX expression did not overtly affect p53 stabilization, however p53 trans-activation function was severely compromised in SS18-SSX expressing cells. U2OS cells expressing SS18-SSX1 were proficient in activating *p21* expression but were defective for the induction of *HDM2, NOXA* and *PUMA* genes. In search for a molecular mechanism we determined that SS18-SSX1 expression enhanced p53 ubiquitination in parallel with cytoplasmic accumulation of p53 when both were expressed in a p53 null cell line. The ubiquitination of p53 induced by expression of SS18-SSX1 could be effectively reduced by siRNA targeting HDM2 implying that SS18-SSX interferes with the p53-HDM2 auto regulatory loop. Interestingly HDM2 protein levels following over expression of SS18-SSX revealed a concomitant increase. Methionine pulse chase analysis showed that HDM2 had a significantly increased protein half-life in the presence of SS18-SSX1, indicating a reduced turnover rate. Next we investigated the molecular mechanism of SS18-SSX1 induced HDM2 stability. Since HDM2 is an intrinsically unstable protein due to its auto ubiquitination activity we analyzed if SS18-SSX expression could alter the

ubiquitination status of HDM2. Our results confirmed a reduction in HDM2 ubiquitination in the presence of SS18-SSX1.

To evaluate the functional consequences of SS18-SSX1 expression on cell survival we exposed U2OS or U2OS SS18-SSX1 expressing cells to numerous cytotoxic agents known to activate p53 apoptotic function. Propidium iodide staining and FACS analysis of DNA fragmentation following exposure to mitomycin C, actinomycin D, doxorubicin or etoposide confirmed that SS18-SSX1 expressing cells had a reduced apoptotic function when compared with parental U2OS cells. Treatment of SS18-SSX1 expressing cells with either siRNA targeting HDM2 or with the Akt inhibitor LY 294002 could partially restore apoptosis induced by mitomycin C or actinomycin D implying that HDM2 is a contributing factor in SS18-SSX1 apoptotic resistance.

The mechanism by which SS18-SSX expression negatively regulates p53 via HDM2 is far from clear. One possible explanation may be due to the gain of function properties of SS18-SSX in promoting the epigenetic de-regulated expression of growth factor genes, particularly those involving the IGF pathway [90]. In such a situation the mis-firing of IFG1R signaling induced by SS18-SSX expression may promote enhanced HDM2 stability via IGF1R mediated Akt activation and subsequent stabilizing phosphorylation of HDM2 [163, 164]. Such a concept has already been shown as a mechanism of decreasing p53 function in breast cancers via the over expression of the Her2/Neu receptors which subsequently promote Akt mediated HDM2 stability [165]. A second and by no means mutually exclusive mechanism is the direct interaction between SS18-SSX and HDM2 or p53 proteins. Interestingly we have recently detected a stress induced interaction between p53 and wild type SS18, which is the larger component the SS18-SSX fusion protein (D'Arcy et al unpublished results). Under such conditions, SS18 a ubiquitously expressed transcription factor may modulate p53 function, however this function may be lost or modified by the fusion of SS18 with SSX in the genesis of synovial sarcoma. The relationship between SS18 and p53 in modulating tumor suppression is an area of ongoing study.

Paper IV

Reactivation of wild type p53 tumor function in synovial sarcomas by inhibition of p53-HDM2 interaction.

The p53-HDM2 loop is a convergence point for numerous pathways and is often deregulated in several tumor types by defects which promote HDM2 over activity, resulting in a reduced p53 response. Consequently targeting the HDM2-p53 regulatory circuit has emerged as a tempting target for the treatment of tumors which retain wild type p53 but loose p53 tumor suppressive function. As a continuation of paper III we investigated the effect of targeting the HDM2-p53 axis with the small molecular weight compound, nutlin 3A in a panel of synovial sarcoma cells expressing endogenous SS18-SSX.

Firstly we tested the ability of nutlin-3A to inhibit cell proliferation in several synovial sarcoma cell lines using the WST-1 proliferation assay. Synovial sarcoma cell with wild type p53 displayed a dose dependent inhibition of cell proliferation when treated with increasing concentrations of nutlin-3A, consistent with the notion that the p53-HDM2 axis is deregulated in synovial sarcoma. Analysis of p53 levels showed increased p53 stabilization which was paralleled by the induction of p53 target genes, *HDM2*, *p21*, *PUMA* and *Bax*. Consistent with the activation of p53 function, analysis of the cell cycle profile following nutlin-3A treatment confirmed the activation of the G1 and G2 checkpoints in synovial sarcoma cells. Analysis of Annexin V staining or DNA fragmentation by FACS analysis showed that nutlin-3A was an effective inducer of p53 dependent apoptosis. Next we compared the nutlin response of synovial sarcoma cells with that of genotoxic agents doxorubicin and etoposide. All three compounds effectively induced p53 stability, however only etoposide and doxorubicin induced p53 Ser 15 phosphorylation. Nutlin-3A treatment effectively induced the trans activation of p53 induced genes, however both doxorubicin and etoposide failed to induce p53 transactivation in spite of the high levels of stabilized p53 protein. Analysis of p53 sub cellular localization following treatment with doxorubicin or nutlin-3A showed the presence of high levels of nuclear p53 in both cell lines with no discernable difference in localization. We investigated the ability of doxorubicin and nutlin-3A to abrogate p53-HDM2 interaction in synovial sarcoma cells using HDM2 immunoprecipitation. Nutlin-3A effectively abrogated p53-HDM2 interaction; however p53-HDM2 complexes were detected following doxorubicin treatment. Analysis of the ability of

low does nutlin-3A to synergize with doxorubicin in promoting p53 dependent growth arrest and apoptosis showed that combinations of both drugs effectively decreased cell viability and promoted apoptosis greater than either treatment alone suggesting a synergistic effect.

The notion that small changes in HDM2 levels can abrogate p53 transactivation has been explored by several groups. Cell lines displaying a polymorphism at position 309 in the first intron of the HDM2 promoter show constitutively enhanced levels of HDM2 transcription and protein expression [152, 153]. Induction of genotoxic stress in these cells failed to disrupt HDM2-p53 interactions, in spite of efficient p53 phosphorylation and stabilization. The HDM2-p53 complexes were nuclear and associated with the chromatin of p53 target genes; however p53 remained transcriptionally inactive, implying that the association of HDM2 with p53 is sufficient to block p53 trans-activation following a stress response. This model is analogous to the data presented in this paper where p53 can be stabilized by DNA damage but remains associated with HDM2 and transcriptionally inactive in synovial sarcoma cells

Thus our results suggest the possibility of using HDM2 inhibitors alone or in combination as a possible therapeutic strategy for synovial sarcoma treatment.

Conclusions & Future perspectives

The present thesis uncovers several new and undescribed oncogenic mechanisms for SSX and SS18-SSX.

Similar to other CT antigens the function of SSX in both normal conditions and in cancer is relatively unknown. In this thesis we provide a link between SSX and stem cell biology. We show that in addition to the traditional sites of expression, several CT antigens families are expressed in normal undifferentiated stem cells. Several findings support the role of SSX in stem cell biology through its interaction with the PcG proteins specifically Bmi-1, a known regulator of stem cell renewal. Our results further strengthen this hypothesis by showing that SSX may be involved in mesenchymal to epithelial transitions by promoting MMP2 expression and cell migration. Although SSX expression is observed in numerous cancers, presumably as a result of massive epigenetic deregulation of *SSX* promoters no concrete correlation establishing an oncogenic role for SSX has previously been described. We also give a proof of concept of the oncogenic function of SSX in promoting tumor cell growth both *in vivo* and *in vitro*.

There is a general consensus that p53 function is lost in virtually all cancers. Since synovial sarcomas have a relatively low number of p53 aberrations when compared with other soft tissue sarcomas we investigated the mechanisms by which SS18-SSX can disable p53 function. Our results show that SS18-SSX can stabilize HDM2 resulting in a two fold effect, namely the enhanced ubiquitination and degradation of p53 under basal conditions and the abrogation of p53 trans activation function under a stress response. In support of this observation targeting HDM2 can efficiently restore p53 transactivation and apoptosis in synovial sarcoma cells suggesting the possibility of targeting the p53-HDM2 axis as a potential treatment strategy for synovial sarcoma.

The investigation into the function of SS18, SSX and SS18-SSX is only beginning. Emerging evidence suggests that SS18 is a mediator of key cellular functions, and SSX is involved in the maintenance of stem cell like phenotype. Study into the pathways regulated by SS18 and SSX extend far beyond their deregulation in synovial sarcoma and may have broader implications in the multi-step process of cancer development.

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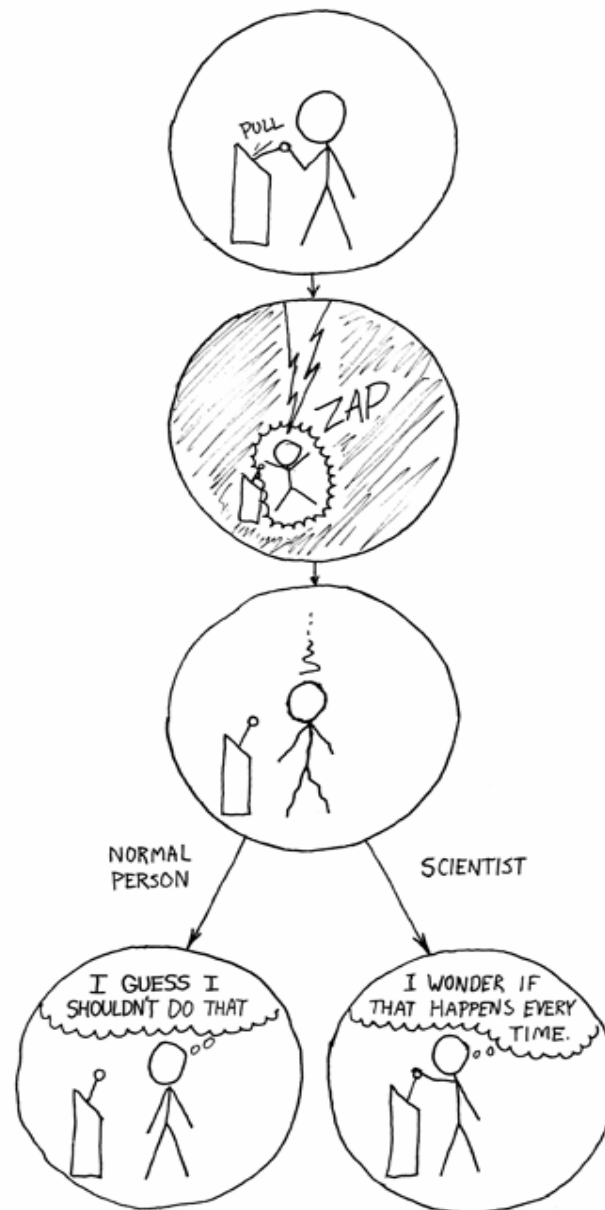
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