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2008 has been another scientifically productive year for the LRI, and also the beginning of significant change for us. Following the publication of the Cancer Research UK five-year research strategy, a major programme of transition began to bring important core research facilities under LRI management for the first time. Plans for the new UK Centre for Medical Research and Innovation continue apace, with LRI scientists working with those from UKCMRI partners in diverse aspects of the planning process.

Cancer Research UK worked intensively during 2008 to finalise its organisational and research strategies for the next five years, and these were published in December 2008 (see http://science.cancerresearchuk.org/research_strategy/). As part of this strategy, CRUK will refocus its scientific support activities round its research Institutes, and close its Research Service directorate. While the Institute’s science infrastructure is thus undergoing significant change, as we review and in some cases restructure scientific core facilities transferring over from Research Services, this is a golden opportunity for us to improve and update our core facilities. Work on the transfer started in late 2008, and over the coming year Julian Downward and Ava Yeo will be working to ensure a smooth completion of the transition.

2008 saw two group leaders move from LRI to new host Institutions, and we wish them all future success. In the summer Ralf Adams moved to the Max Planck Institute for Molecular Biomedicine in Münster, Germany, where he will broaden his focus to tissue morphogenesis in general. In October, Ian Tomlinson moved to the Wellcome Trust Centre for Human Genetics in Oxford. Ian’s work has focussed on the genetic epidemiology of cancer; which has been revolutionised by highthroughput genomic approaches over the last decade: Ian has been a leader in the identification of common gene variants associated with significantly increased cancer risk, and he reviews his research at the LRI in the front section of this Report. The successful review of Banafshe Larijani’s Biophysics group was deservedly recognised by her promotion to Senior Staff Scientist. Two new core facility groups formally joined LRI during the year; including Protein Production, under Svend Kjaer; and Bioinformatics, under Aengus Stewart.

It is always a pleasure to record professional acclamations accorded to LRI scientists. At Clare Hall, Tomas Lindahl received the French Institute of Health and Medical Research (INSERM) award for his work on the molecular mechanisms of DNA repair; Steve West gave the 2008 Biochemical Society Novartis award lecture, Simon Boulton received the Eppendorf award for young cancer researchers, and Helle Ulrich was elected to EMBO. At Lincoln’s Inn Fields, Caetano Reis e Sousa received the 2008 Life Sciences award from the Lilian Bettencourt Foundation, while Erik Sahai was awarded the British Society for Cell Biology’s 2008 Hooke medal. Jonathan Leslie, a postdoc in Julian Lewis’ group, where also he conducted his PhD research, was awarded the 2008 Werner Risau prize of the German Society for...
for Cell Biology, while amongst our graduate students, Andreas Pichlmair from Caetano Reis e Sousa’s group was awarded the Pontecorvo Prize, for the best CRUK-funded thesis of 2007.

While LRI is well funded through the generous core support by Cancer Research UK, its scientists also make efforts to supplement this funding through applications to outside granting and fellowship agencies. Amongst our successes in this regard during the year, Holger Gerhardt was a recipient of a prestigious Lister Prize research grant, while Vincenzo Costanzo received a Starting Investigator grants in the first round of European Research Council awards.

2008 saw the second LRI Symposium, on Chromosome Biology, organised by Frank Uhlmann, Julie Cooper and Simon Boulton. These small discussion meetings bring together leaders in the field, LRI group leaders and staff, and external participants to provide a detailed overview of an area of research of interest to LRI. Our graduate students attended the second European Cancer Research graduate student conference, held in Amsterdam, and will organise next year’s conference which will be held at the Institute. An LRI postdoc retreat was held in February, with a focus on scientific collaboration, and featuring the CRUK Chief Scientist David Lane as a keynote speaker. A novelty as this meeting was a ‘science speed-dating session’ designed to promote interaction between groups, which was judged a scientific (and social) success by all concerned.

Since the foundation of LRI following the establishment of CRUK in 2002, we have experimented with different formats for our annual research conference, usually jointly with other CRUK-funded institutes or centres. While providing an outside perspective, these formats have precluded attendance by all LRI scientific staff. We broke from this tradition this year and held an all-inclusive LRI retreat at the University of Kent in Canterbury, organised by John Diffley and Nicola Hawkes. Half our research groups gave oral presentations at the meeting, with the rest presenting posters, an arrangement that will be reversed next year. A varied social programme was capped the LRI’s very own Olympic Games organised by Facundo Batista, in which teams of group leaders, postdocs and students battled to better each other at everyday and not-so-everyday laboratory activities: perhaps fittingly for the future of the Institute, the coveted Golden-Gilson award for the best team was won by the students.

During the year the UKCMRI project has begun to take concrete shape, with acquisition of the British Library site, and the appointment of architects and engineers. The development of the design brief for the building has been commenced in consultation with LRI scientific and other staff, with a planning application due in the coming year. The UKCMRI Science Planning Committee (SPC), led by Paul Nurse, has been taking forward the development of the detailed UKCMRI strategy for research and science governance. During the year the SPC made the landmark recommendation that the partners should work together towards the ultimate aim of a single institute, pursuing a fully-integrated research strategy, through a single management board and budget. As a first step towards convergence of the two Institutes’ strategies, group leaders from the LRI and NIMR participated in an enjoyable retreat in Hampshire in October. The full development of the UKCMRI strategy, and the mechanism for transition into the new Institute upon its completion in 2014, will be big challenges for the future.

Richard Treisman
Creativity is not a virtue that is commonly associated with the cancer researcher on those few occasions that the public mind turns to thoughts of science. The output of the cancer researcher is judged as dutiful, even perfunctory, and by its practical benefits. This is a shame, because of the pleasures inherent in scientific research, trying to create, usually unsuccessfully, is chief amongst them. One of the virtues of the London Research Institute is the ability to try new, risky things. In my group over the past 10 years, we have made new mouse models that would not have been possible in the programme grant-based University environment. We have also successfully identified genes that cause cancers that were not thought to be genetic, by spending time and money on collecting and characterising cases and their families. At one point, we were even planning to identify a major gene that controlled melanism in the two-spot ladybird! Perhaps that was a project too far, but if it had succeeded, the scientific insights would have been profound.

If the LRI has allowed my colleagues and me to try to be creative, my own area of research – cancer genetics – has been moving in the opposite direction. Research in human genetics has been drifting steadily away from smaller, but thoughtful, studies to much, much bigger screens for new disease genes. But the bigger science becomes, the more expensive it is, and the less the scope for being creative, because deviating from the standard protocol becomes too risky.

My group’s most high-profile work has recently been to search for common genes that increase the risk of the cancer. My collaborators and I have been reasonably successful in this regard. We have needed tens of thousands of patient samples for this work, representing huge efforts for several members of my laboratory and the laboratories of collaborators. But it has to be admitted that the main developments that have made this work possible are technological – undertaken by large genotyping organisations rather than research groups such as mine – and financial, owing to the willingness of Cancer Research UK to fund what remains very expensive work. I shall describe some of this work in order to show what genetics has become, for better or worse, and to show what might become of other specialties that currently follow the creative/risky model of science.

**Big-scale genetics and the genome-wide association study**

Over the past 2 years, my group and colleagues at the Institute of Cancer Research and in Edinburgh...
have been searching for common alleles that increase the risk of developing colorectal cancer. The primary justification for these activities is that by fully understanding the genetic architecture of cancer, we can predict individual risk and thus tailor effective cancer prevention measures to those at relatively high risk. A secondary justification is that we will obtain important information about disease biology, by identifying some of the functional pathways that might usefully be targeted by, for example, new chemopreventive agents. The project design has been that of a relatively simple association study, based on comparing genotype frequencies in cases and controls and searching for significant differences at individual loci.

The history of association studies to identify genes that increase the risk of the common diseases has been mixed at best, with the norm having previously been small, underpowered studies based on candidate genes. Admittedly, there have been some successes, such as the HLA loci and other genes related to the immune response in inflammatory disorders, but the situation in the common cancers has been particularly bleak in that very few candidate genes, however well chosen, have been linked to differential susceptibility. The existence of recombination hotspots, and hence blocks of the genome characterised by strong linkage disequilibrium, has meant that a hypothesis-free screen for common cancer predisposition alleles has become not only possible, but routine, on a genome-wide basis in the last 3 years. Large commercial panels of several hundred thousand so-called tagSNPs can cover almost the whole genome, based on the associations of tagSNPs with other SNPs in the same haplotype block.

Despite a couple of false starts, the hypothesis-free method has proven much more successful than the candidate gene approach. For colorectal cancer, for example, we have so far identified 10 tagSNPs that are associated with differential risks of disease. In one case, the SNP is also associated with increased risks of prostate and ovarian cancer; but the other 9 SNPs seem unique to colorectal cancer. In all cases, the effects on risk are relatively modest, with typically a 1.1-1.3-fold differential risk per allele (although these estimates are likely to be conservative, as the effect of the causal variant(s) will typically be larger.
than the association detected through a tag SNP). These SNPs seem to act independently of one another, with no instances to date of gene-gene interactions and little deviation from additive or log-additive effects on risk. Perhaps most intriguingly, there is emerging evidence that several of the colorectal cancer SNPs act in the same functional pathway, namely bone morphogenetic protein (BMP) signalling: two SNPs are located close to or within BMP2 and BMP4; one is adjacent to the secreted BMP antagonist Gremlin1; and yet another is within the inhibitory SMAD, SMAD7. Recently, inhibition of BMP signalling has independently been proposed as having an essential function in maintaining the stem cell niche at the bottom of the colonic crypt. If one accepts the view that cancer arises from normal stem cells, it is therefore possible that the colorectal cancer SNPs affect stem cell numbers, and hence the number of cells that potentially give rise to cancer. More stem cells may provide some advantages, but presumably would increase cancer risk. Other interesting, albeit negative, findings from the study are that none of the SNPs appear to act in the Wnt pathway that is central to normal crypt homoeostasis and early colorectal tumorigenesis, and none lies in pathways involved in DNA repair or maintenance of genome integrity. Furthermore, several colorectal cancer SNPs lie in regions where genes in the conventional sense are absent; thus, whilst the true cancer-causing variants remain unknown, we know that few, if any, of them alter protein sequence and the most likely function is regulation of gene expression, potentially over hundreds of thousands of kilobases. Finally, the corpse of the candidate gene-based approach is still twitching, as one of the colorectal cancers SNPs is within the prime candidate gene E-cadherin and had been detected by previous studies.

What happens next to these large-scale genetic studies? Some new analyses, such as copy number variation assays, are feasible with current samples. However, in most respects, the scientific imperative is that the studies change a little, but essentially become even larger. In this way, we hope to move from tagging SNPs to the real disease-causing variants, to identify additional common susceptibility alleles that confer even lower risks, to take the work into different human populations and ethnic groups, and to identify rarer, perhaps ‘private’ susceptibility alleles that confer modest risks, but that cannot be found using a linkage disequilibrium-based approach. Without this additional investment, however, history may not judge the genome-wide association studies in colorectal and other cancers to have been successes, because we remain some way from describing the genetic architecture of the common cancers.

The alleles identified so far can only explain a small proportion of the total genetic variation in cancer risk. Hence, although our SNPs can predict differential risks of colorectal cancer of up to 5-fold on the population level, they currently have little predictive value for the individual. Therefore, whilst Cancer Research UK hold patents on the cancer-associated SNPs we have found, it is probably too early to use these SNPs for predictive genetic testing. This is not a universal opinion, however: a little disturbingly, some organisations are already attempting to market tests for the panels of cancer SNPs and, presumably, they will make specific recommendations for cancer screening based on the results. With its major influence on cancer policy, Cancer Research UK has a crucial role to play, and must provide leadership in addressing the ethical questions arising from our increasing ability to test, screen and predict outcomes for the cancer patient.

The genome-wide association study has thus taken genetics into new areas in which the science has become much more similar to epidemiology than ‘classical’ genetics. Scientists must by their nature welcome change, but what is happening feels a lot like de-skilling. The detective work and clinical acumen that have been needed to study rare, Mendelian cancer syndromes are largely irrelevant when studying common-or-garden cancers. Largely missing too are the interesting and illuminating quirks associated with the rare syndromes, such as the genotype-phenotype associations and the specific somatic genetic pathways followed by each cancer type. In the place of these factors has come the requirement for
The plots show the genomic regions around 4 of the SNPs associated with differential bowel cancer risk. The upper plots in each case show strengths of association under the allelic test (Y axis) for each SNP typed in the discovery Phase 1 of our study (red), or in Phases 1 and 2 (replication) combined (blue), or in all Phases (yellow) as a function of genomic position (X axis, NCBI build 36.1). The lower plots show the linkage disequilibrium structure in each region, with recombination hotspots (between haplotype blocks) shown as green bars. Lines connect the most strongly associated SNP in each case with its position within a haplotype block.
project management, co-ordination of colleagues and a great deal of time spent in assembling and running large international consortia. These factors come together to dilute the contribution made by each person within the now-very large team, to move the research away from a model based on the drive of individuals, and hence to reduce personal satisfaction.

Implications for the London Research Institute and the UKCMRI

Granted that the intellectual satisfaction in identifying a couple of handfuls of new bowel cancer genes is relatively small, should I not simply be satisfied that what we have done is potentially a big step forward and of importance for human health? The answer has to be “Yes, but ….” I suspect, moreover, that most of my ex-colleagues at the LRI would agree. Whilst I cannot see the LRI developing any affection for the sorts of studies being undertaken today in disease genetics, no area of molecular biology is safe from the erratic advance of the large-scale, hypothesis-free study. The trick for the LRI – and its successor, UKCMRI – in the future must be to balance clinically important research against risky, creative research and to ensure that there is sufficient payback in terms of personal satisfaction that researchers of all types and ages remain motivated. Evidently, each research group does not need to fulfil all of these criteria, but the Institute as a whole perhaps should do so. In recent history, the LRI has succeeded very well in the latter two categories by focussing on basic science of the highest quality, but it has placed a lesser emphasis on clinically-oriented research.

In many ways, this strategy makes sense. The LRI approach is essentially to take a scientific problem, broadly within the field of biology, and to poke it hard with a ‘molecular stick’ so as to disturb the biological system or pathway of interest. By disrupting the normal biological order in this way, highly specific insights into highly specific areas of biology have been achieved. Piecing these insights together requires great skill. Clinical research cannot, by its nature, take this approach; it is more scythe than stiletto. Moreover, since the demise of the Imperial Cancer Research Fund, LRI groups can no longer feel any direct affiliation to or, just as accurately, easily influence over Cancer Research UK-funded groups working in clinical settings. Access to patient samples is no longer straightforward for LRI groups and even where samples are available, outside groups act as gatekeepers. Finally, most clinically-based research, not only Genetics, is going down the bigger-is-better route, contrary to the LRI philosophy.

There is, however, a downside to the current LRI approach. There is more than a little truth in the contention that having some groups working on patients and their cancers, whilst intellectually less exciting, has the benefit of continually bringing the thoughts of the basic science groups back to the clinical problems. How might this be achieved while building on the LRI’s undoubted strengths? I see three potential ways forward.

One possible change would be to recruit more group-leader scientists and clinician scientists whose primary interests were in areas directly related to cancer patients. The jobs should be made sufficiently attractive for the latter such that their clinical careers were not threatened. At the very least, it should be ensured that some of the LRI groups focus on genes and proteins – or specific cellular processes – that we know to be important for tumour growth. For new groups, it could specifically be stipulated that they should study the genetic and other changes actually found in cancers, rather than simply focussing on model systems that are amenable to analysis, but probably do not represent the changes found in real tumours. These new groups need to be given access to high-throughput methods, through third party providers if necessary. This may require a change in the historical balance of LRI laboratories, such that the preponderance of Post-Docs over Scientific Officers is reversed in these groups.

A second change might be to introduce a pot of competitive funding to allow buy-in of LRI groups to
external collections of cancer patients and tumours. The intention of this funding would be to encourage highly promising changes found in the basic science laboratory to be examined in real tumours. The bar would have to be set high in terms of scientific justification.

Third, the LRI could grasp the nettle and use its scientific standing and power to train some young medically-qualified researchers to be the next generation of clinician scientists. In doing so, however, there needs to be some tiered system of qualification and a long period of assessment, such that only those truly fitted for and committed to academic research proceed to the highest level. No doubt, this idea will rankle with some, who seem blind to the problems and sheer waste created by having too many Clinical Fellowships, especially given the recent retrograde steps that have created even more hybrid junior doctors/researchers. However, the LRI has the ability to lead in this field and to make a difference to UK academic medicine as a whole.

The establishment of the new UKCMRI provides an opportunity to introduce such changes, in a research environment that will bring together LRI, the MRC’s National Institute for Medical Research, and UCL, with additional funding by the Wellcome Trust. UKCMRI will certainly have the ability to attract the best researchers. However, the devil, as ever, is in the detail, especially as regards institute structure and recruitment policy, and it is to be hoped that big does not mean immobile or unimaginative. I would urge the UKCMRI Science Planning Committee, which is developing the strategy for the new Institute, to introduce schemes of the sort outlined above to generate an Institute with a unique focus on the biology of human health and disease.

At least the LRI has some choice about its future. The Cancer Geneticist has no choice but to cuddle up to the philosophy of the big study and the large collaborative consortia that accompany it. Science demands these changes, but having tried to adapt my group’s work in cancer genetics to the LRI philosophy over a period of years, I now find it odd to shift to the more perfunctory and managerial skills required to run studies that involve large patient cohorts. I suspect that this fate eventually awaits most of those sub-disciplines within cancer research that are currently regarded as high-skill and bespoke; there are many signs of it already in activities such as those that aim to create conditional knock-out mice for every gene or to knock-down every gene transcript. In the meantime, in my group, the large-scale studies will go on for as long as there is funding available, partly because we very much do wish to do clinically useful work. Quietly, however, I regard part of their attraction as providing seedcorn for the functional studies that they will engender; so that we can work out exactly what are the differences between individuals that cause variation in cancer risk and to work out how these differences have their effects. Without 10 years at the LRI, my group would have no expertise in anything other than human genetics, and we would be unable to move beyond the discovery phase of genetic studies. At the risk of sounding mawkish, my conclusion must be a positive one: the scientific opportunities that the LRI has provided me will always influence my work and I have been very lucky to have worked in such an environment.

Selected references


Research Highlights

Here we feature significant research advances made by Institute scientists, summarising the findings in terms accessible to the nonspecialist scientific reader. The winner of the Hardiman-Redon Prize, awarded to a junior researcher who has made outstanding contributions to a highlighted publication during the year, is chosen from amongst these papers. In a departure from the usual sole recipient, the 2008 prize is awarded jointly to Stephen Ip, Ulrich Rass, and Miguel Gonzalez-Blanco from Steve West’s lab at Clare Hall, for their work on the identification of the Holliday junction resolvase.

For bowel cancer, we know of 10 genes that affect the risk of disease in the general population. This report describes two of those genes. In one case, the variation probably affects a protein called EIF3H that is involved in initiating protein production. In the other, the variation is in a region with no known genes that make proteins, and the cause of the increased cancer risk remains mysterious. Typically, it is possible to predict a 20-40% increased risk of bowel cancer, other things being equal, as a result of the two genes.


The amoeboid movement of cancer cells requires contraction and co-ordination of the cortical acto-myosin network. To learn more about how cortical actin is regulated Sophie Pinner performed an siRNA screen of cytoskeletal regulators including all Rho family GTPases, adhesion molecules, many regulators of actin polymerisation, and a range of signal transduction molecules. This work identified an unexpected role for the kinase PDK1 in controlling cortical acto-myosin. Sophie further demonstrated that PDK1 is important for amoeboid cancer cell motility *in vivo* and initial metastatic colonisation of the lungs. The molecular mechanism by which PDK1 function is also somewhat surprising: kinase activity is not required but instead PDK1 binds to ROCK1 and prevents its association with the negative regulator RhoE. This promotes the ability of ROCK1 to regulate the phosphorylation of myosin light chain and the cell cortex and thereby generate contractile force and enable cell movement.

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It is becoming increasingly apparent that common, inherited differences between individuals can influence their risks of many diseases. Identifying the genes involved can help us to work out what are drives cancer development and may allow tests to be develop to identify those at high risk of cancer.
Identification of Holliday junction resolvases from humans and yeast. Ip SC, Rass U, Blanco MG, Flynn HR, Skehel JM, and West SC. Nature 2008;456:357-361 (Figure 1).

In 1964 Robin Holliday proposed a model for genetic recombination that accounted for the various types of products that are formed during meiosis in fungi. Central to this model was the presence of a four-way DNA intermediate that links homologous chromosomes, a structure also thought to be formed during the recombinational repair of DNA double-strand breaks. To complete recombination, and allow the chromosomes to separate in preparation for cell division, these Holliday junctions need to be resolved by nucleolytic cleavage. Enzymes that carry out this 'scissors' function have been identified from bacteriophage, bacteria and archaea, but it has been extremely difficult to identify analogous enzymes from human cells. However, after an 18-year search, the human Holliday junction resolvase was finally identified as the product of the GEN1 gene. The identification of this protein is a significant advance in understanding the mechanism of recombinational repair, a process that is critical for the maintenance of genome integrity and tumour avoidance.

The regulated assembly of a PKCe complex controls the completion of cytokinesis. Sarin AT, Durgan, Cameron AJ, Amir Faisal, Marber MS and Parker P. Nat Cell Biol. 2008;10 (8):891-901 (Figure 2).

Various controls operate during the course of the cell cycle to ensure a precise and orderly duplication process. This paper defines a very late regulatory mechanism in the progression of cell division operating at the final stage of daughter cell separation, abscission. Through a combination of knock-down, chemical-genetic and mutational approaches it is shown that the serine/threonine protein kinase, PKCe, is required for efficient completion of cytokinesis. The catalytic activity of the kinase is shown to be required and activation is imposed through the assembly of a PKCe/14-3-3 complex. The downstream target of PKCe/14-3-3 is the GTPase RhoA, which is involved in contraction of the actin ring at the division furrow. RhoA needs to be switched off in order...
for completion of cytokinesis – when PKCε is dysfunctional/absent Rho remains active. Thus PKCε has a controlling input very late in cell division to permit appropriate completion of cytokinesis and avoid a polyploid outcome.

Mathematical modeling identifies Smad nucleocytoplasmic shuttling as a dynamic signal-interpreting system. Schmierer B, Tournier AL, Bates PA, Hill CS. Proc Natl Acad Sci U S A. 2008;105(18):6608-13 (Figure 3).

This paper describes the first highly constrained kinetic model of the TGF-β/Smad pathway. The model explains how Smad phosphorylation, Smad complex formation, Smad nucleocytoplasmic shuttling and Smad dephosphorylation act together as a system to transduce TGF-β signals into the nucleus. Importantly, our model conclusively demonstrates that Smad nucleocytoplasmic dynamics function as a signal-interpreting mechanism. This feature of intracellular Smad signalling has major implications. Many ligands of the TGF-β superfamily act as morphogens, and cell fate decisions depend on the signal strength received by a target cell. The accurate and dynamic transmission of changing signal strengths into a corresponding amount of transcriptionally active, nuclear Smad complexes is key for the dynamic interpretation of a morphogen gradient. We convincingly demonstrate that Smad nucleocytoplasmic dynamics can perform this task.

SREBP activity is regulated by mTORC1 and contributes to Akt dependent cell growth. Porstmann T, Santos RS, Griffiths B, Cully M, Wu M, Leevers S, Griffiths JR, Chung Y-L, Schulze A. Cell Metab. 2008;8(3):224-36 (Figure 4).

Cell growth requires coordinated activation of processes that provide metabolites for the synthesis of macromolecules such as proteins and lipids. This study shows that the PI3-kinase/Akt/mTORC1 signalling pathway, which has been implicated in the regulation of protein biosynthesis and is frequently activated in cancer, regulates the expression of enzymes required for lipid biosynthesis by activating the SREBP transcription factor. We could show that SREBP was required for the induction of cell growth of mammalian tissue culture cells in vitro and for cell and organ growth in the model organism Drosophila melanogaster. These results suggest that the regulation of lipid biosynthesis lies downstream of a cellular signalling pathway that integrates growth factor signalling with cellular energy status and nutrient availability. Our work also demonstrates that protein and lipid synthesis are regulated in a concerted manner during cell growth.

Fission yeast Ccq1 is telomerase recruiter and local checkpoint controller. Tomita K and Cooper JP. Genes Dev. 2008;22:3461-3474

The linearity of eukaryotic chromosomes poses two problems; first, the conventional replication machinery cannot fully copy the termini of linear DNA molecules. Second, cells
need to distinguish chromosome ends from damage-induced DNA breaks, which trigger checkpoints that arrest cellular growth. To solve these problems, shortened chromosome ends engage telomerase to add telomeric DNA repeats to the end of the chromosome. In turn, these repeats protect chromosome ends from triggering checkpoints. Shortened telomeres lose this protective ability. How short telomeres ‘decide’ whether to engage telomerase or trigger checkpoints remains a great mystery. In this paper, we show that the Ccq1 protein is crucial for managing this decision. Ccq1 is required to recruit telomerase to chromosome ends, and to prevent moderately short telomeres from triggering cell cycle arrest. Hence, Ccq1 acts at those telomeres whose shortening means their checkpoint suppression activity is most precarious to ensure their replenishment by telomerase.


Blood vessels formation and function is essential for embryonic development and physiology. Growing tissues provide signals, stimulating endothelial cells to make vascular tubes that sprout, connect and remodel into an intricate hierarchical network.

The vascular endothelial growth factor (VEGF-A), a key stimulant of blood vessel formation, regulates multiple

Figure 5. Confocal laser scanning micrograph of cephalic vessels of a normal mouse embryo labelled with antibodies against the endothelial protein endomucin (red), illustrating formation of a hierarchical pattern of large and small calibre vessels.
aspects of endothelial behaviour including migration of leading endothelial tip cells and proliferation of endothelial stalk cells during blood vessel sprouting. Both responses are triggered by activation of the same tyrosine kinase receptor VEGFR2. How specificity of VEGFR2 signalling is achieved, and which downstream signalling components control divergent functions is poorly understood. This study reveals a previously unanticipated degree of selectivity of phosphoinositide 3-kinase (PI3K) p110 subunits in regulating distinct aspects of endothelial cell behaviour during blood vessel formation, providing a possible mechanism for divergent downstream functions of VEGFR2 tyrosine kinase activity in endothelial tip and stalk cells.


Poly(ADP-ribose)ation is a post-translational modification that involves the addition of long chains of ADP-ribose units to target proteins, and is critical for a wide range of fundamental processes including DNA repair; regulation of chromosome structure, transcriptional regulation, mitosis and apoptosis. This paper identifies a novel zinc finger motif, a Poly(ADP-ribose)-Binding Zinc finger (PBZ), in a number of eukaryotic proteins involved in the DNA damage response and checkpoint regulation. We demonstrate interaction of poly(ADP-ribose) with this motif in two representative human proteins, APLF and CHFR. We also show that the PBZ and PAR metabolism are both required for the function of CHFR in the antephase checkpoint. Since CHFR is frequently mutated in human epithelial cancers our findings provide molecular insights into the role of CHFR and the antephase checkpoint in cancer.


Homologous recombination (HR) is an accurate method of DNA repair critical for genome stability. Inappropriate recombination is known to cause chromosomal abnormalities and tumorigenesis, yet the mechanisms that restrain recombination are very poorly understood. The helicase Srs2 is known to antagonize HR in yeast, but functional analogues in higher eukaryotes have proven to be elusive despite 30 years of research. This paper identifies C. elegans RTEL-1 as a functional analogue of Srs2 and describes its vertebrate counterpart, RTEL1, which is required for genome stability and tumour avoidance. We show that worms and human cells deficient for RTEL1 share characteristic phenotypes with yeast srs2 mutants. Furthermore, purified human RTEL1 counteracts HR by promoting the disassembly of a specific recombination intermediate. We propose that deregulation of recombination is the cause of genomic instability in Retel knockout mice and this may drive tumorigenesis as RTEL1 is amplified in some human tumours.


Many of the chemotherapies currently in clinical use work by interfering with DNA replication, either by damaging DNA, depleting precursors of replication or directly inhibiting replication enzymes. Weakening of DNA damage checkpoints is believed to occur early in oncogenesis and may provide an important therapeutic window because checkpoint deficient cells are hypersensitive to these chemotherapies. Using budding yeast as a model, we found that loss of a single gene, EXO1, almost completely suppresses the sensitivity of the rad53 checkpoint mutant to a wide variety of DNA damaging agents. This identifies a novel mechanism by which cancer cells might develop resistance to chemotherapies. We found that deletion of EXO1 does not suppress the loss of the upstream
checkpoint protein kinase, Mec1, and that a second downstream kinase, Chk1, can act to stabilise replication forks in the absence of Rad53 and Exo1. These results indicate that checkpoints preserve stalled DNA replication forks by multiple mechanisms.


The chromosomal condensin complex is among the three most abundant chromosomal protein components and gives mitotic chromosomes their shape and stability. By using genomic microarray technology and budding yeast as the model organism, we characterise for the first time the chromosomal addresses of the condensin complex. This leads us to the discovery of a loading factor for condensin that associates with a subset of highly transcribed genes and defines the condensin binding pattern. Our results reveal an intriguing relationship between mitotic chromosome condensation and the chromosome’s transcriptional landscape between mitotic cell divisions in interphase. We are now taking advantage of this information to study the architecture of mitotic chromosomes.


As chromosomes are replicated in every S-phase of the cell cycle, the two replication products (the sister chromatids) are linked to each other by a protein complex called cohesin. In order that cohesin can hold sister chromatids together after their synthesis, the enzyme Eco1 is required which has the ability to acetylate its target proteins. We have in the past shown that Eco1 is part of the replication machinery that moves along chromosomes to duplicate them in S-phase, but what the acetylation target of Eco1 was and how its acetylation would promote sister chromatid cohesion remained unknown. Now, we show that Eco1’s acetylation target is the Smc3 subunit of the cohesin complex, and that Eco1 acetylates Smc3 as chromosome replication progresses. The consequence of Smc3 acetylation is that cohesin becomes resistant to destabilisation by a protein called Wapl. Resistance to Wapl in turn is crucial for cohesin to be able to provide stable sister chromatid cohesion.

Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. Sancho D, Mourão-Sá D, Joffre OP, Schulz O, Rogers NC, Pennington DJ, Carlyle JR, Reis e Sousa C. J Clin Invest. 2008;118:2098-2110 (Figure 8).

Dendritic cells (DC) present antigens to T cells to initiate acquired immune responses. Therefore, the selective delivery of antigens to DC can be used to improve vaccination against infectious diseases and cancer. Sancho et al. identified DNGR-1 as a new surface marker of DC in mouse and human and showed that it can be used for antigen targeting in a mouse model. Upon injection into mice, monoclonal antibodies to DNGR-1 selectively bound to DC and were endocytosed by the cells. Antigens coupled to those antibodies were then processed by the DC and presented on MHC class I molecules to CD8+ T cells. When those antigens corresponded to tumour-restricted peptides, the approach could be used to elicit potent cytotoxic T cell responses that were able to mediate eradication of growing transplanted melanoma tumours. Thus, targeting antigens to DNGR-1 is a promising approach for therapeutic cancer vaccination.

Figure 7. Chromosome condensation in budding yeast. Two fluorescently marked loci (MMP1-GFP and YLR003c-GFP) in 137 kb distance from each other on the left arm of chromosome 12 were visualised in cell in interphase and mitosis. The two loci are seen as clearly separate in interphase, but come close together as the consequence of chromosome condensation in mitosis.

Figure 8. Caetano Reis e Sousa is pioneering the use of DNGR-1 as a target for delivering antigens to dendritic cells.
Exploring the dynamics of chromosome structural maintenance proteins by elastic networks

The study of structure-function relationships for biological macromolecules, and the complexes they form, is crucial to our understanding of cellular activities. However, the dynamics and the functioning mechanism for many protein complexes are still not clear. Therefore, computational techniques that take account of available structural and biochemical information, are necessary to elucidate how bio-molecules work together. Coarse-grained elastic network models, that use a mathematical technique called normal mode analysis, are computationally efficient models for determining the collective dynamics of macromolecules in the cell. Here, crystal structures of the macromolecules can be modelled at lower than atomic resolution, enabling us to simulate macromolecular dynamics on much longer time courses than with classical molecular dynamics approaches. The elastic network models are currently being used to explore the dynamics of the supramolecular complexes of cohesin and condensin; chromosome structure maintenance proteins that play important roles during cell division. The molecules are similar in structure yet different in function; cohesin is responsible for holding two sister chromatids together during cell division whilst condensin facilitates promotion of mitotic chromosome condensation by reorganising chromosomes into their compact shape. All insight derived from the dynamic interplay between these proteins and the chromosomes they affect will be tested by further experimentation in collaboration with Frank Uhlmann (Chromosome Segregation Laboratory). The work should greatly enhance our understanding of sister chromatids cohesion and chromosome condensation processes at the molecular level.

Simulating protein-protein interactions in signal transduction pathways

Atomic-level simulations of protein-protein interactions usually only consider two proteins at a time. However; this is unrealistic as in vivo bimolecular interactions take place in an environment where there are many proteins interacting simultaneously (Figure 1). We are developing a computer simulation package (BioSimz) that uses a simulation technique called Brownian Dynamics. Using our program we can study how signal transduction proteins, such as Ras and GAP interact at the atomic-level in the crowded protein environment of the cytosol. One of the key questions we are...
trying to answer is: does an overcrowded environment facilitate the interaction between the relatively low concentrations of signal transduction proteins or inhibit such interactions? Moreover, for mutations within these proteins, how do the protein network dynamics of interaction change?

Our computer simulations indicate that molecular crowding cannot be ignored if we are to understand the full consequences of signalling strengths and how they are modulated in tumourogenesis.

**Multiscale modelling of cell motility**

Cell migration is an essential component in many biological processes including metastasis in cancer. Recent studies suggest that, depending on the cell type and environmental conditions, the mechanisms of cellular morphology and motility can vary significantly. Understanding these variations is important since inhibiting metastasis is as essential as minimising tumour growth for efficient treatment of cancer. In our current multiscale computer model of cell motility, the primary focus is on amoeboid type cell motility of metastasising tumour cells in the extracellular matrix (ECM). Both the extracellular conditions (e.g. ECM density) and intrinsic cell properties (e.g. relative distribution of contractile and blebbing regions of the cell membrane) are being investigated. In collaboration with Erik Sahai (Tumour Cell Biology Laboratory), experimental data on cell morphology during motion is being utilised in both the construction and validation of the computational model. At a later stage, the model will be extended to include mesenchymal types of cell motility, and according to availability of kinetic and qualitative data on proteins involved in regulation of the modelled process, protein interaction networks will also be implemented.

**Simulating cell-cell interactions during angiogenesis**

In collaboration with Holger Gerhardt (Vascular Biology Laboratory) we have develop a novel three-dimensional agent-based multiscale model ‘the memAgent model’ to investigate the dynamics of tip cell selection by Notch-Dll4 lateral inhibition in normal and pathological angiogenesis (blood vessel formation). The model predicts tip cell selection failure in pathological angiogenesis. We now believe this to be due to a synchronous oscillation of Dll4 in high VEGF environments. Modelling also highlights a more important role for filopodia and shape changes in selection than previously thought.

Ongoing research into the later stages of angiogenesis, utilising a novel inclusion of membrane physics into the model in the form of springs between agents, has revealed unexpected insights into the robustness of tip/stalk cell patterning. The model predicts that cell fates will flip upon anastomosis and that junction size, between cells, contributes to the robustness of the system in pathological environments by naturally regulating Dll4/Notch binding between neighbouring cells.

The model has highlighted a possible new avenue for normalising tip selection in pathologically high VEGF environments, via partial inhibition of the actin pathway alone. This is shown to reduce filopodia, which in turn reduces surface area and junction size through reduced migrational stretching. These reductions lead to lower Dll4 production and presentation at junctions, which helps stabilise selection in high VEGF environments.

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**Publications listed on page 121**
Antigen-induced B cell activation

B cell activation is initiated in response to the recognition of specific antigen through the surface B cell receptor (BCR). The BCR is a complex consisting of a heterotetrameric membrane immunoglobulin (mIg), together with a membrane embedded Igα/β sheath. Antigen engagement of the BCR triggers the phosphorylation of tyrosine residues within the Igα/β leading to the recruitment and activation of a plethora of intracellular signalling molecules and adaptors. These molecules are extensively organised such that they can co-ordinate cellular responses such as cytoskeleton rearrangements and induction of gene expression. One established consequence of B cell activation in response to membrane-bound antigen is the reorganisation of proteins within the plasma membrane. This reorganisation allows BCRs to concentrate in a central cluster and act as a platform for antigen internalisation, allowing the subsequent recruitment of T cell help and maximal activation of B cells.

Microclusters of BCRs organise intracellular signalling and orchestrate B cell spreading

In previous work we showed that during activation, B cells rapidly spread across antigen-containing surfaces, prior to a more prolonged contraction phase (Fleire et al., Science, 2006). This two-phase cellular response has important consequences as it allows the B cell to accumulate greater amounts of antigen, and thus shapes the outcome of B cell activation. In order to elucidate the molecular mechanism underlying the B cell spreading response, we have examined an extensive panel of knock-outs generated in the DT40 B cell line (Weber et al., J Exp Med. 2008). This screen, verified in primary cells, established an important role for the kinases Lyn and Syk, and for intracellular effectors and adaptors such as Vav, PLCγ2 and Blnk. Subsequently high-resolution TIRFM was used to visualise the spatiotemporal dynamics of these effectors within the B cell. In response to membrane-containing antigen, we observed the rapid formation of BCR-microclusters throughout the contact area, in agreement with our previous observations (Depoil et al., Nat Immunol. 2008). These microclusters are the sites for the sequential recruitment of Lyn and Syk, and coordinate the subsequent assembly of numerous ‘microsignalosomes’, we suggested that they might be the common signalling units in lymphocytes. Furthermore, we detected cooperation between PLCγ2 stickies and Vav, components of the ‘microsignalosome’, such that each enhances the recruitment and retention of the other (Weber et al., J Exp Med. 2008). Interestingly, this offers a molecular explanation for the essential role of CD19 in mediating B-cell spreading and activation in response to membrane-bound antigen (Depoil et al., Nat Immunol.)
As the cytoplasmic domain of CD19 contains binding sites for numerous intracellular signalling molecules, CD19 through transient association with BCR-microclusters can mediate the recruitment of additional molecules of Vav and PI3K. Thus CD19 can enhance signalling through the BCR in response to membrane-bound antigen, propagating spreading and thus facilitating B cell activation.

iNKT cells and TLR9 stimulation can mediate the formation of extrafollicular plasma B cells

Following antigen-induced activation, B cells can differentiate along two alternative pathways. The first of these is important in early immune responses and results in the formation of extrafollicular plasma cells capable of the rapid production of low-affinity antibodies. In contrast, the second involves entry to the germinal centre for affinity maturation, and yields plasma cells able to secrete extremely high affinity antibodies and memory cells that confer long-lived protection. However at this stage, the factors responsible for determining the differentiation pathway and thus shaping B cell fate remain poorly characterised. To shed light on aspects of this complex process, we have designed and employed particulates directly conjugated with both antigen and other immunostimulants as an investigative tool. As these particulates cannot be taken up by B cells through phagocytosis, they provide a method for selectively targeting the activity of the immunostimulant to specific B cells. We observed that immunisation with particulates containing antigen and αGalCer, an iNKT cell stimulatory ligand, resulted in the enhanced formation of antigen-specific extrafollicular plasma cells (Barral et al., PNAS, 2008). A dissection of the mechanism underlying the observed enhancement revealed that BCR-mediated particulate internalization was required such that αGalCer could be loaded onto endosomal CD1d. This internalization was dependent on the avidity of the BCR-antigen interaction surpassing a tightly regulated threshold. The subsequent presentation of αGalCer-CD1d on the B cell surface allowed for the recruitment of iNKT cell help, resulting in the formation of extrafollicular plasma cells capable of class-switched antibodies. In a similar manner, we have observed that immunization with particulates containing antigen together with a TLR9 ligand generated enhanced formation of antigen-specific extrafollicular plasma cells (Eckl-Dorna and Batista, in press). Thus these particulates have aided the identification of factors intrinsic and extrinsic to the B cells that can influence the outcome of B cell differentiation.

Future directions

Taken together, research in the Lymphocyte Interaction Laboratory within the past year has made a considerable contribution to the understanding of the processes underlying B cell activation. In spite of our significant progress a number of challenges remain, namely the characterisation of the dynamics and regulation of cytoskeleton reorganisations during B cell activation and the determination of factors responsible for shaping B cell fate in vivo. We are seeking to address these, and other, issues through the development and application of innovative high-resolution imaging techniques including single-particle tracking and multi-photon microscopy.

Publications listed on page 121
Regulation of intestinal homeostasis and tumourigenesis by JNK signalling

Wnt signalling is a key signalling pathway controlling intestinal homeostasis and cancer. We have recently found that the Jun N-terminal kinase (JNK) MAP kinase pathway and one of its most important substrates, the AP-1 transcription factor c-Jun, modulates Wnt signalling strength in the intestine. Transgenic gut-specific augmentation of JNK signalling stimulated progenitor cell proliferation and migration, resulting in increased villus length. In the crypt, c-Jun protein was highly expressed in intestinal stem cells (ISCs) (Figure 1a) and absence of c-Jun resulted in decreased proliferation and villus length. In addition to several known c-Jun/AP-1 target genes, expression of Wnt targets genes Axin2 and Lgr5 were stimulated by JNK activation, suggesting a crosstalk of JNK to Wnt signalling. Expression of the Wnt pathway component TCF4 was controlled by JNK activity and chromatin immuno-precipitation and reporter assays identified \textit{tcf4} as a direct c-Jun target gene. Consequently, increased JNK activity accelerated tumourigenesis in a model of colorectal carcinogenesis. Since \textit{c-jun} is a direct target of TCF4/β-catenin, the control of \textit{tcf4} expression by JNK/c-Jun leads to a positive feedback loop that connects JNK and Wnt signaling (Figure 1b). This mechanism regulates the physiological function of progenitor cells and oncogenic transformation.

Molecular mechanism of phosphorylation-dependent c-Jun-mediated neurodegeneration and cancer

AP-1 activity is strongly induced in response to numerous signals including growth factors, cytokines and extracellular stresses. AP-1 stimulation is mediated, in part, by the phosphorylation of c-Jun by the JNKs. c-Jun N-terminal phosphorylation (JNP) at the serine residues 63 and 73 and threonine residues 91 and 93 within its transactivation domain is thought to increase transcription of target genes, one of which is the c-jun gene itself. Genetic inactivation of c-Jun N-terminal phosphorylation by a knock-in approach demonstrated an essential role for JNP in stress-induced neuronal apoptosis and tumorigenesis. As the JNK/c-Jun pathway is required for clinically important diseases including neurodegenerative insults and cancer, mediators of JNP may be promising candidates for therapeutic intervention.
To investigate the mechanism of JNP-mediated transcriptional regulation, we have identified proteins that interact with c-Jun in a phosphorylation-dependent manner. Several candidate proteins that preferentially interact with N-terminally phosphorylated c-Jun (Phosphorylation-dependent c-Jun interactor (PDJ) 1, 3, 4 and 5) or that bind preferentially to the unphosphorylated form of c-Jun have been identified (PDJ2).

We have recently shown that PDJs are novel disease-relevant regulators of the JNK pathway. For example, PDJ3 encoded the tumor suppressor Fbw7, and Fbw7 was found to be an E3 ubiquitin ligase specific for phosphorylated c-Jun. Another PDJ encoded the transcription factor TCF4 and our studies revealed that the interaction of phosphorylated c-Jun with TCF4 is essential for intestinal cancer development.

Preliminary characterisation revealed that other PDJs have several different biological activities, ranging from transcriptional cofactors to chromatin regulators. Currently we are using biochemical methods and gene targeting in mice to understand the functions of PDJs in neurodegeneration and cancer. The combination and integration of the biological activities of phosphorylation-dependent interactors of c-Jun have the potential to elicit basically any physiological response depending on the cellular context and could be an important mechanism underlying cell-type specificity of MAP kinase signalling.

**ATMIN defines a novel pathway of ATM signalling**

ATM is a member of the phosphatidylinositol kinase-related protein family that includes ATR and DNAPKcs. These kinases respond to the presence of DNA damage or replication blocks by activating cell cycle checkpoints and promotion of DNA repair. ATM is mutated in the genomic instability syndrome ataxia telangiectasia (A-T), which is characterised by problems in motor coordination, immunodeficiency and increased tumour incidence. We have recently identified a novel essential cofactor for ATM, which we named ATMIN (for ATM interacting protein). ATMIN interacts with ATM through a carboxy-terminal motif, which is also present in another ATM cofactor called Nijmegen breakage syndrome 1 (NBS1). ATMIN and ATM colocalised in response to ATM activation by chloroquine and hypotonic stress, but not after induction of double-strand breaks by ionizing radiation (IR). ATM/ATMIN complex disruption by IR was attenuated in cells with impaired NBS1 function, suggesting competition of NBS1 and ATMIN for ATM binding. ATMIN protein levels were reduced in A-T cells and ATM protein levels were low in primary murine fibroblasts lacking ATMIN, indicating reciprocal stabilisation. While phosphorylation of Smc1, Chk2 and p53 was normal after IR in ATMIN-deficient cells, basal ATM activity and ATM activation by hypotonic stress and inhibition of DNA replication was impaired. Thus ATMIN defines a novel NBS1-independent pathway of ATM signalling.

Figure 1. Function of JNK/c-Jun in intestinal stem cells and cancer. a) c-Jun protein is expressed in ISCs (indicated by arrowheads) and staining is absent in gut-specific c-jun knock-out mice (c-junΔG). b) Model illustrating a positive feedback loop that connects JNK and Wnt signaling.

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Relationship between CD34neg and CD34pos cells

A few years ago, others and we have identified a novel class of murine and human haematopoietic stem characterised by its lack of CD34 expression (Bhatia et al., Nat. Med. 1998; 4: 1038-1040). One of our objectives is to study the relationship between CD34+ and CD34+ stem cells. We found recently using serial transplantation that CD34+ cells are more primitive than CD34- cells. The CD34+ cells represent a quiescent reservoir of stem cells. We went on to look at what were the molecular mechanism explaining the quiescent of these CD34+ cells. We investigate the Notch, Wnt, TGFbeta pathways. We show that Notch4 is highly express on these cells and that Delta4 expressed by placenta keep the cells in an undifferentiated stage and have no role in cell cycle control. We also find that the truncated form of Lef (Delta LEF) is highly expressed and that these cells do not respond to WNT3A in vitro. TGF beta is also highly expressed and we are at present studying the role of this pathway in the regulation of CD34- cells (Fernando Anjos-Afonso et al., Manuscript in Preparation).

Leukaemic stem cell

Immunodeficient mice are increasingly used to assay human hematopoietic repopulating cells as well as leukaemia initiating cells. One commonly used method to isolate these rare cells is to sort cells stained with fluorochrome-conjugated antibodies into fractions. The different fractions are then transplanted into immunodeficient mice to test their repopulating ability. The antibodies are generally treated as being neutral in terms of their effects on the experiment. Human repopulating cells are thought to express CD34 and lack CD38. Recently we show that anti-CD38 antibodies have a profound inhibitory effect on engraftment of cord blood and leukaemia cells. We show that this effect is Fc-mediated and can be overcome by treating mice with immunosuppressive antibodies. When this inhibitory effect is prevented, we demonstrate that the CD34+CD38+ fraction, of certain acute myeloid leukaemia samples, contains all or at least most leukaemia initiating cells capacity. We show using mice pre-treated with anti-CD122 that in some patients, LSCs could have a progenitor phenotype (CD34+/CD38+). Based on the heterogeneity of AML in terms of karyotype, differentiation stage of the blasts, and clinical outcome; it is not surprising that LSCs is more complex than previously.
thought and can vary from patient to patient and also probably in the same patient depending on the stage of the disease. This heterogeneity not only indicates a potential differential origin or progression of the disease but also have important implications in the development of new therapies to eradicate these cells (Taussig et al. 2008).

**Leukaemic stem cells and their microenvironment**

Over the years, we have been trying to purify LSCs and test their repopulating activity using our xenotransplantation model. It appears that like normal HSC, leukaemia might be dependent on microenvironment clues. Thus, we have developed way using two-photon microscopy to visualise and describe the location and nature of this niche. To our surprise, we show that both calvaria and long bones are highly vascularised and thus the notion of a separate vascular and osteoblastic niche should be revisited (Figure 1). Further studies are now aiming at describing the composition of the niche in both normal and leukaemic situation.

**Stem cell from the stroma system of the bone marrow**

A couple of years ago we prospectively characterise the mesenchymal stem cells (MSC) compartment present in mouse bone marrow. We found that these stroma cells are organised as a hierarchy and that a small fraction of these cells are capable of differentiation at least in vitro into seven different lineages (Anjos-Afonso F and Bonnet D. Blood, 2007; 109: 1298-1306). Because Oct4 and Nanog expression seem to be correlated with multilineage capacity we recently investigate the role of Oct4 and Nanog in MSC development. Surprisingly, the overexpression of Oct 4 in human mesenchymal stroma cells induced cell death and senescence signals. This effect can be overcome by Nanog overexpression. Thus it appears that Oct4 is not responsible for the multipotentiality of MSC and by itself act as an apoptotic/senescence signal (Bithiah Grace et al. Manuscript in Preparation).

**Stem cells as a vehicle for gene and cell therapy**

In collaboration with Dr Sam Janes, we evaluate the therapeutic potential of Bone marrow Derived Stem Cells (BMDSCs) and determine their ability to produce a sustained delivery of KGF to the lungs in an attempt to ameliorate pulmonary fibrosis through an increase in epithelial proliferation. For that purpose, we used the BLM-induced lung fibrosis model and the use of BMDSCs as therapy vehicles. We demonstrated, that this combined cell/gene therapy not only is able to deliver KGF to the injured lung parenchyma, but KGF protects from BLM-induced lung injury. We report that KGF induces proliferation of alveolar type II cells and decrease pro-inflammatory cytokines and collagen levels within the lungs. Finally, comparing MSCs and HSCs as a therapy vectors, our data suggests that HSCs are greater gene therapy vehicles able to ameliorate lung fibrosis (Susana Aguilar et al. Manuscript in Preparation).

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**Figure 1. Vascularisation of long bone**

- Growth Plate
- Metaphysis
- Diaphysis
- Bone
- Endothelial cells
Telomeres are also critical for solving the ‘DNA end replication problem’, the inability of conventional DNA polymerases to replicate the extreme ends of linear DNA molecules. Telomeres solve this problem by engaging telomerase, a specialised reverse transcriptase containing an internal RNA subunit which templates synthesis of telomere repeats. In humans, telomerase is expressed in germ cells but not in most somatic cells. However, tumor cells must activate telomerase or an alternative mode of maintaining telomeres. While loss of telomere function promotes early tumorigenesis, the genomic instability that stems from telomere loss is incompatible with long-term cellular survival. Therefore, regeneration of telomeres is critical for the eventual ‘immortalisation’ of cancer cells and suggests an intriguing universal target for anti-cancer therapy.

Fission yeast telomeres have a protein complement similar to that found in humans, but present substantial experimental benefits. Pot1 binds the terminal single strand telomeric overhang. Taz1, the only known ortholog of human TRF1 and TRF2, binds double strand telomeric DNA and regulates numerous functions including telomerase-mediated synthesis and nearby transcriptional silencing. It prevents DSB repair reactions from acting inappropriately on chromosome ends -- loss of Taz1 leads to lethal chromosome end-fusions during G1 when nonhomologous end-joining (NHEJ) activities are elevated but not during G2 when NHEJ levels are low. Hence, telomere dysfunction yields strikingly different outcomes during G1 versus G2, conferring an advantage to studying telomeres in fission yeast, whose mainly G2 cell cycle allows cells lacking Taz1 to grow despite their dysfunctional telomeres.

Control of telomerase through the cell cycle
Our recent studies shed light on how the telomere complex engages and regulates telomerase. Taz1 prevents accumulation of stalled telomeric replication forks (see below), which are themselves potent stimulators of telomerase activity. However, we find that telomerase engagement is possible only when Pot1 is phosphorylated during S-phase. We also find that the protein Ccq1 is crucial for telomerase recruitment. In the absence of Ccq1 or Pot1 phosphorylation, telomeres fail to restrain local checkpoint activation and telomeric recombination. These observations provide a foothold for deciphering the molecular underpinnings of cell cycle control of telomere accessibility to both telomerase and DNA damage response factors.

Control of replication fork movement through telomeres
While telomerase synthesize the most terminal telomere repeats, most telomeres are maintained by the activity of telomerase-independent DNA replication mechanisms. We are investigating a model for telomere elongation in G1, in which Ccq1-dependent DNA synthesis extends previously shortened telomeres. This model is supported by evidence that in fission yeast, telomeres with dysfunctional Ccq1 or Pot1 can be successfully elongated by telomerase, whereas telomeres lacking functional Taz1 cannot recover through telomerase activity.

The ends of eukaryotic linear chromosomes are potentially dangerous sites, as their resemblance to damage-induced DNA double strand breaks (DSBs) makes them vulnerable to DNA degradation and end-joining pathways. If left unchecked at chromosome ends, these pathways cause chromosome shortening and rearrangement, which in turn provoke genomic instability and cancer. Telomeres protect chromosome ends from these hazards. We study the components of telomeres, the spectrum and mechanisms of telomere function, and the events that follow telomere loss.

Telomeres are also critical for solving the ‘DNA end replication problem’, the inability of conventional DNA polymerases to replicate the extreme ends of linear DNA molecules. Telomeres solve this problem by engaging telomerase, a specialised reverse transcriptase containing an internal RNA subunit which templates synthesis of telomere repeats. In humans, telomerase is expressed in germ cells but not in most somatic cells. However, tumor cells must activate telomerase or an alternative mode of maintaining telomeres. While loss of telomere function promotes early tumorigenesis, the genomic instability that stems from telomere loss is incompatible with long-term cellular survival.
repeats, the majority of telomere repeats are synthesised by semi-conservative DNA replication. We found that Taz1 is required for efficient replication fork movement through telomeres, as stalled replication forks accumulate at telomeres lacking Taz1. We find that such fork stalling is conferred by all repeated sequences and may have general relevance for genome maintenance. Replication fork stalling at telomeres leads to abrupt telomere loss in the absence of telomerase. It also leads to telomeric ‘entanglements’ that prevent chromosomes from segregating properly at mitosis. Our search for factors involved in resolving this telomeric entanglement has uncovered two surprising key players. First, the RecQ helicase Rqh1 (ortholog of human Werner Syndrome helicase, whose mutation causes the eponymous premature aging disease) prevents resumption of fork movement through telomeres, triggering telomere loss and entanglement. Second, a non-canonical activity of the essential decatenation enzyme Topoisomerase II (Top2) prevents the entanglement that can result from stalled telomeric replication forks, heralding an unforeseen role of Top2 in promoting genome stability.

A novel mode of survival in the absence of telomeres
When telomeres are lost in telomerase-minus cells, some cells acquire the ability to maintain telomeres via recombination. In addition, fission yeast can survive telomere loss by chromosome circularisation. These ‘circular strains’ are viable but exhibit several conspicuous defects, like slow growth and hypersensitivity to agents that induce DSBs. We have identified a DSB-resistant subclass of telomerase-minus cells that survive using a third strategy, in which telomere sequences are absent but large blocks of heterochromatin are amplified. This survival mode resembles that found in the fruit fly Drosophila, and may illuminate the universal ‘stripped-down’ requirements for chromosome end-maintenance.

Telomeric control of meiotic spindle formation
When cells progress to the meiotic cell cycle, telomere function changes dramatically. Telomere clustering during early stages of meiosis, or ‘bouquet formation’, is observed throughout the Eukaryota. Our earlier studies showed that formation of this telomere bouquet depends on Taz1 and is required for successful meiosis. We find that telomeres not only associate with the centrosome during meiotic prophase, but also dissociate in a concerted manner, at a moment that immediately precedes centrosome division and the onset of meiosis I. In the absence of the telomere bouquet (e.g. in taz1 Δ cells), the centrosome mislocalises at meiosis I and often dissociates from the nucleus. Moreover, meiotic spindle formation is aberrant. Thus, the highly conserved bouquet plays an unanticipated role in controlling spindle formation. Our data suggest that an ectopically engineered association between non-telomeric heterochromatin and the meiotic centrosome can confer proper meiotic spindle formation in the absence of a true ‘telomere bouquet’. These data raise exciting new questions about the control of spindle formation by chromosomes.
Investigation of mechanisms of transformation by Ras oncogenes

Much of the work in the laboratory has focused on the RAS family of oncogenes and the signalling pathways that they control. RAS genes are activated by point mutation in some 20% of human tumours and are known to play a key role in the establishment of the transformed phenotype. While the early signalling pathways activated by Ras are now well characterised and the transcriptional programmes they induce have been documented in detail using microarray technology (e.g. Schulze et al., Genes & Development 2001; 15: 981 and Schulze et al., Mol. Biol. Cell 2004; 15: 3450), it remains a major challenge to understand later events in oncogene-induced signalling and, in particular, which regulated genes are important in the establishment of the transformed phenotype. In order to investigate novel aspects of these pathways in cancer cells, especially those with activated RAS oncogenes, we have employed a functional genomics approach using post-transcriptional gene silencing by genome-scale libraries of RNA interference agents.

Two approaches to screening have been applied. In one, genes corresponding to a large fraction of the genome are systematically silenced one by one, allowing identification of genes required for a particular aspect of the transformed phenotype. An example of a successful high throughput screen from the lab published recently (Swanton et al., 2007 Cancer Cell; 11: 498) studied the effect of knock down of expression of different genes on the sensitivity of cancer cells with activated RAS oncogenes to common chemotherapeutic agents. This has lead to the identification of proteins that might be potential therapeutic targets for overcoming resistance of tumours to existing drugs. In particular, targeting a ceramide transport protein, COL4A3BP/CERT, results in multidrug sensitisation in several tumour lines, with less effect on untransformed cells, suggesting that it could be a promising chemosensitisation target. The suitability of CERT as a drug target is currently being investigated in collaboration with Cancer Research Technology.

In the second approach – a selective screen using retroviral RNA interference vectors – many genes are silenced at the same time in a mixed pool of cells, with cells only surviving that acquire the desired phenotype as a result of knock down of a specific gene. These cells, along with the RNAi sequence they carry, are then identified as they emerge at the end of the screen. An example of this approach was published previously (Nicke et al., Molecular Cell 2005; 20: 673) in which we identified MINK, a MAP4 kinase acting in the p38 stress activated protein kinase pathway, as a protein required for RAS oncogene induced senescence in ovarian epithelial cells. A novel adaptation of this methodology allows the identification of shRNA sequences lost from a population of cells under selective pressure by the use of high throughput sequencing of barcodes in the shRNA library to give a digital readout of library sequence representation. This
methodology is being used currently in the lab to address the mechanisms whereby tumour cells acquire resistance to targeted therapeutic agents such as small molecule inhibitors of EGF receptor and IGF-I receptor tyrosine kinases, a phenomenon that represents a major limitation of novel therapies in the clinic.

A whole genome-scale screen has now been completed searching for synthetic lethal interactions between gene silencing and activation of the Ras oncogene, comparing a colon cancer cell line containing an activated KRAS allele with a normal derivative in which this has been deleted by homologous recombination. This has uncovered proteins whose therapeutic targeting might be expected to provide differential toxicity towards tumour versus normal cells. The relationship between synthetic lethality with Ras oncogene activation and oncogene addiction to activated Ras is also being studied with hits from this screen.

**The role of phosphatidylinositol 3-kinase in Ras-driven oncogenesis**

Ras proteins signal through direct interaction with a number of effector enzymes, including type I phosphatidylinositol (PI) 3-kinases. Although the ability of Ras to control PI 3-kinase has been well established in manipulated cell culture models, evidence for a role of the interaction of endogenous Ras with PI 3-kinase in normal and malignant cell growth in vivo has been lacking. We have generated mice with mutations in the Pi3kca gene encoding the PI 3-kinase catalytic p110α isoform that block its ability to interact with Ras (Gupta et al., 2007 Cell; 129: 957). Cells from these mice show proliferative defects and selective disruption of signaling from certain growth factors to PI 3-kinase. The mice also display defective development of the lymphatic vasculature due to reduced signalling from VEGF-C to PI 3-kinase. Most importantly, the mice are highly resistant to endogenous KRAS oncogene induced lung tumourigenesis and HRAS oncogene induced skin carcinogenesis. The interaction of Ras with p110α is thus required in vivo for certain normal growth factor signaling and for Ras-driven tumour formation. The demonstration of the importance of the Ras/PI 3-kinase interaction in tumourigenesis raises the prospect that agents that disrupt this interaction might have particular value in cancer therapy.

This work is being further pursued by the generation of mice with inducible expression of the inactivating mutation in the Ras binding domain of p110α so that the requirement of this interaction for tumour maintenance, rather than simply tumour initiation and development, can be assessed. In addition, the effect of this mutation in PI 3-kinase on tumourigenesis driven by other oncogenes acting upstream of Ras, such as EGF receptor, is also being studied.

**Publications listed on page 122**
During angiogenesis, vascular networks form through guided sprouting, branching and establishment of new connections between sprouts. The density of the network is a result of the initial branching frequency and subsequent regulated regression. Our recent work illustrates that two conserved signalling pathways, the Notch and the Wnt pathway, are coordinated in endothelial cells at vascular branch points by the small ankyrin repeat protein Nrarp, and that this function is required to balance proliferation and maintain vessel stability during network formation. Together, Notch and Wnt-signalling determine whether to establish or release new vessel connections by regulating sprouting, proliferation and cell-cell contacts.

**Make or break vascular connections**

Blood vessel formation is triggered by physiological demands of metabolically active tissue. Undersupply, in particular of oxygen, leads to transcriptional activation of multiple genes that will help cells survive oxygen shortage, while initiating a cascade of blood vessel changes to re-establish adequate supply.

Hypoxic cells produce the vascular endothelial growth factor (VEGF-A), which stimulates formation of new vessel sprouts that branch, elongate and make connections to establish perfusion. Effective sprouting requires selection of an endothelial tip cell that will extend filopodia to probe the environment for the VEGF-A gradient (Gerhardt et al., 2003 JCB 161, 1163-1177). Migration speed of the tip cell is controlled by a gradient of VEGF-A. Our recent studies illustrate that the formation of new tip cells is the default response of endothelial cells to the stimulation by VEGF-A gradients (Bentley et al., 2008 J Theor Biol 250, 25-36; Hellstrom et al., 2007 Nature 445, 776-780). In order to form a functional sprout, this default response is inhibited in cells adjacent to the tip. These endothelial cells instead form the stalk cells, establish adhesion junctions and polarise to form the lumen. The tip cells inhibit their neighbours by producing Dll4, a transmembrane ligand of the Notch receptor. Dll4 activates Notch signalling, which suppresses sprouting (Hellstrom et al., 2007). Experimentally, ectopic stimulation of Notch signalling in endothelial cells inhibits sprouting, whereas a loss of Notch signalling leads to excessive sprouting through increased tip cell formation.

The Notch-regulated ankyrin repeat protein (Nrarp) is induced in endothelial cells by Dll4/Notch signalling (Phng et al., 2009 Developmental Cell in press). Nrarp expression is most prominent in the stalk cells and at branch points in nascent vessels. To address the function of Nrarp, we studied the effects of morpholino-based knockdown of Nrarp in zebrafish embryos and retinal angiogenesis in Nrarp-deficient mice. Loss of Nrarp function causes a substantial decrease in vessel density although vessel sprouting at the level of tip cells is not affected. Instead, reduced vessel density is caused by dynamic regression and loss of connectivity in newly formed vessels. Time-lapse confocal microscopy in zebrafish illustrates that vessels fail to maintain their connections in the absence of Nrarp. This failure is associated with reduced stalk cell proliferation. Vessels thus contain fewer endothelial cells, which nevertheless strive to bridge the same distance, leading to excessively thin, and fragile connections (Figure 1).

At the molecular level, Nrarp controls Notch signalling through a negative feedback loop. Nrarp binds and interacts with the transcriptional activator complex consistent of the
Notch intracellular domain (NICD), histone acetylases and the DNA binding molecule Rbpjk (also known as CSL). Nrarp destabilises this complex and leads to NICD degradation, thus limiting Notch signalling (Figure 2).

Inhibition of Notch signalling in the absence of Nrarp can restore vessel density through induction of new vessels. However, these new vessels also remain fragile and regress, suggesting that Nrarp performs additional functions to stabilise vessels.

Previous work in zebrafish neural crest development identified that Nrarp also interacts with the transcription factor lymphoid enhancing factor (Lef1) (Ishitani et al., 2005 Nat Cell Biol 7, 1106-1112). Also in endothelial cells, Nrarp binds Lef1 and increases its transcriptional activity in the canonical Wnt signalling pathway (Phng et al., 2009). Studying localisation and quantitative signalling activity in vivo and in vitro, we find that Nrarp and Wnt-signalling reporter co-localise in stalk cells, while loss of Nrarp suppresses endothelial Wnt-signalling. Downstream targets of Wnt-signalling include the cell cycle regulator cyclin D1. Whereas notch signalling suppresses cell proliferation through retinoblastoma protein de-phosphorylation and down-regulation of p21CIP (Noseda et al., 2004 MCB 24, 8813-8822), Wnt-signalling promotes proliferation (Masckauchan et al., 2006 Physiology Bethesda, Md 21, 181-188) through up-regulation of cyclinD1 (Shutman, M et al., 1999 PNAS 96, 5522-5527). The dual control of Notch and Wnt by Nrarp provides a mechanism for endothelial cell proliferation control. This may also explain why endothelial stalk cells readily proliferate although they receive strong Notch activation.

The nature and origin of the Wnt-ligands involved in this process remain to be determined. Premature vessel regression in mouse mutants of Lrp5 and following endothelial deletion of β-catenin suggest that the canonical Wnt-pathway is the key regulator of vessel stability (Phng et al., 2009). The Notch and Wnt-signalling components identified in our research are induced under hypoxia and VEGF-A stimulation, suggesting that these pathways are mainly functional in endothelial cells during patterning of the angiogenic response.

Hypoxia responses, VEGF-A up-regulation, and formation of new blood vessels are important events during tumour progression and ischemia induced neo-vascularisation processes. It is tempting to speculate that a similar mechanism involving Notch and Wnt-signalling regulates vessel stability during adult neo-vascularisation. We therefore hope that our identification of the mechanism required for stability of newly formed blood vessels will open new possibilities to modulate blood vessel formation and function in disease.

Publications listed on page 122
A mathematical model to describe TGF-β/Smad signalling

The best understood signalling pathway downstream of receptors for TGF-β superfamily members is the Smad pathway. In essence, ligand stimulation induces receptor activation which leads to the phosphorylation and activation of a subset of R-Smads, for example, Smad2 and Smad3 in the case of TGF-β/Activin/Nodal ligands. Activated R-Smads then form homeric and heteromeric complexes with Smad4 that accumulate in the nucleus, where they are directly involved in regulating transcription of target genes. However, it has become apparent from our experimental work over the past several years that the TGF-β/Smad signal transduction pathway is not a simple linear, unidirectional pathway from receptors to the nucleus. Instead, it is a dynamic network where the Smads constantly shuttle between the cytoplasm and nucleus both in the absence and presence of a signal, the latter being driven by successive rounds of Smad phosphorylation in the cytoplasm and dephosphorylation in the nucleus. In this model, nuclear accumulation of active Smad complexes results from a change in mean residence time of the Smads in nucleus versus the cytoplasm. To address whether this experimental model can account for the observed kinetics of Smad nuclear accumulation in response to signal and their redistribution to the cytoplasm upon receptor inactivation, we have developed a computational model of the TGF-β/Smad signalling pathway in collaboration with Paul Bates’ lab, using four distinct kinetic datasets generated in our lab. The resulting highly-constrained mathematical rate equation model simultaneously fits all the datasets with excellent accuracy and in doing so, verifies the plausibility and mechanistic relevance of the underlying network topology. We have used the model to make predictions about the outcome of fluorescence recovery after photobleaching experiments and the behaviour of a functionally-impaired Smad2 mutant, which we have experimentally verified. Most importantly, our computational model clearly demonstrates a functional role for Smad nucleocytoplasmic shuttling in the dynamic and quantitative interpretation of TGF-β signals, which can explain how TGF-β superfamily ligands act as morphogens (Schmierer et al., 2008, PNAS, 105, 6608-6613) (Figure 1).

E3 ubiquitin ligases in TGF-β superfamily signalling

In the last few years we have used high-throughput siRNA screening to discover new components and regulators of TGF-β/Smad signalling, and have identified two E3 ubiquitin ligases, Arkadia and Ectodermin, that play crucial roles.
Following on from our publication in 2007 that Arkadia is an essential component of the Smad3-dependent branch of TGF-β/Activin/Nodal signalling which functions by inducing ligand-dependent degradation of the transcriptional repressor, SnoN, we have focused this year on understanding the relevance of this for cancer progression and for early vertebrate development, using zebrafish as a model system. We have identified a lung tumour cell line which harbours a homozygous nonsense mutation in the Arkadia gene, which generates a non-functional protein. Stable reintroduction of wild-type Arkadia into this cell line restores TGF-β-induced SnoN degradation, Smad3-dependent transcription and substantially reduces the ability of these tumour cells to grow in soft agar. This suggests that Arkadia may be a novel tumour suppressor gene and we are currently investigating this in more detail by generating a conditional mouse knockout of Arkadia and searching for Arkadia mutations in other tumour cell lines. In contrast to Arkadia, Ectodermin acts as a negative regulator of TGF-β/Activin/Nodal signalling pathways. We have shown that its repressive activity requires its PHD and Bromo domains, and consistent with the ability of such domains to bind modified histones, we have shown that Ectodermin is recruited to promoters of TGF-β target genes in a ligand-dependent manner. We are currently investigating how this is achieved and how Ectodermin functions as a repressor.

**Two highly-related PP2A regulatory subunits exert opposite effects on TGF-β/Activin/Nodal signalling**

Our high-throughput screening also led us to the identification of two highly-related PP2A regulatory subunits, Bα and Bδ as important modulators of TGF-β/Activin/Nodal signalling. In a collaboration with Laurel Raftery (MGH, Boston, USA) and using a combination of experimental systems (tissue culture cells, Xenopus and Drosophila embryos) we have shown that Bα enhances TGF-β/Activin/Nodal signalling by stabilising basal levels of type I receptors, whereas Bδ negatively modulates these pathways by inhibiting receptor activity (Batut et al., 2008, Development, 135, 2927-2937).

**A novel branch of TGF-β/Smad signalling is required for anchorage-independent growth**

Contrary to the original view of the TGF-β superfamily signalling whereby TGF-β/Activin/Nodal stimulation leads to activation of Smad2 and 3, whereas BMP/ GDF signalling is mediated via Smad1, 5 and 8, we have now discovered that TGF-β also strongly induces phosphorylation of Smad1 and 5 in epithelial cells and fibroblasts. This signalling requires TβRII, ALK5, and additionally the type I receptors ALK2 and/or ALK3. Simultaneous activation of the R-Smads, Smad2/3 and Smad1/5 results in the formation of novel mixed R-Smad complexes containing, for example, activated Smad1 and Smad2, which we propose are responsible for transducing the signal to the nucleus. Finally, we have shown that Smad1/5 activation by TGF-β is not required for growth inhibition, but is specifically required for TGF-β-induced anchorage-independent growth, suggesting that this branch of TGF-β/Smad signalling may play an important role in tumour promotion (Daly et al., 2008, Mol. Cell. Biol. 28, 6889-6902).
The behaviour of LFA-1 on migrating T lymphocytes: signaling and other intracellular processes that control migration

The integrin LFA-1 is a promigratory adhesion receptor that immune cells utilise at many stages of an immune response. For example, when cells are recruited from the circulation, they use LFA-1 to migrate across the blood vessels into lymph nodes and infected tissues. How the integrin directs this migration has been a main focus of interest for the Leukocyte Adhesion Laboratory. We have discovered that different conformations of LFA-1 are organised into several distinctive activity zones on the membrane of migrating T cells. LFA-1 with high affinity for ligand ICAM-1 is located in the mid-cell region and this ‘focal zone’ is maintained by linkage to the cytoskeletal protein talin (Smith et al., J. Cell Biol. 170:141-151, 2005).

Recently, Paula Stanley found that LFA-1 of intermediate affinity forms new adhesions at the leading edge or lamellipodium of the T cell (Stanley et al., EMBO J. 27, 62-75, 2008). This conformation of LFA-1 interacts with the actin binding protein, α-actinin-1, and disruption of this link dissolves the LFA-1 attachments at the leading edge resulting in loss of cell spreading and migration. These different conformations of LFA-1 must cooperate for the cell to migrate. Our model is that high affinity LFA-1 attachments in the focal zone provide stability and support for intermediate affinity LFA-1 at the leading edge while the T cell scans surfaces, such as lymph node associated blood vessels. This study highlights a biological function for two active conformations of LFA-1 for the first time.

When T cells use LFA-1 to attach to ICAM-1, the resulting ‘outside in’ signalling causes cells in suspension to adhere and then migrate. We aim to dissect the signalling pathway that is immediately downstream of LFA-1. Lck and ZAP-70 are the main Src and Syk tyrosine kinase homologues in T cells and Rachel Evans finds that both are physically associated with LFA-1. These kinases are also essential for LFA-1-mediated migration as demonstrated by the use of pharmacological inhibitors and knockdown of Lck and ZAP-70. Rachel is interested in discovering where in the migrating T cell this signalling pathway operates and whether it is associated with a particular conformation of LFA-1.

Although our models of immune cell migration are very LFA-1-dependent, there may be other receptors involved. It is well known that the chemokines that stimulate the migration of naïve T cells signal through heterodimeric G proteins of the Gαi class. Lena Svensson finds that T lymphoblasts are able to migrate randomly on ICAM-1 without direct chemokine
that Kindlin-3 enables talin to bind to integrin, but much remains
restores adhesion and migration. There is evidence to suggest
lymphocytes with Kindlin-3 cDNA, but not CalDAG-GEF1
expression. Importantly, transfection of the patients’
result in decreased Kindlin-3 mRNA and loss of protein
mutations in the FERMT3 gene in Maltese and Turkish patients
(Svensson
Kindlin-3 protein, as the cause of the LAD-III disorder
mutations in the nearby FERMT3 gene, that specifies the
responsible for the LAD-III disorder. Instead we have identified
Svensson, Alison McDowall and Irene Patzak in a collaboration
that is important for integrin activation. However Lena
LAD-III candidate protein as it activates the GTPase Rap-1
11q13.1 that codes for the protein. CalDAG-GEF1 is a good
C>A mutation in the CalDAG-GEF1 gene on chromosome
51-60, 2003). The LAD-III lesion has been attributed to a
characteristically have mutations in the
The functional importance of integrins has been highlighted
Leukocyte Adhesion Deficiency
The functional importance of integrins has been highlighted by
Leukocyte Adhesion Deficiency-1 (LAD-1) patients who
characteristically have mutations in the β2 subunit of the
leukocyte integrins. The resulting lack of membrane-
expressed β2 integrins on leukocytes is directly linked to
recurrent bacterial infections experienced by the patients.
The platelet integrin αIIbβ3 initiates the process of blood
clotting through binding to fibrinogen and mutation in either
α or β subunit of αIIbβ3 leads to the bleeding disorder;
Glanzmann thrombasthenia (GT). About 10 years ago an
additional group of patients, named LAD-III, was identified
that display symptoms of both LAD-1 and GT. Unlike these
disorders, the haematopoietically-derived cells of the LAD-III
patients express normal levels of the β1, β2 and β3 integrin
subsets, but these integrins fail to function because of
defective ‘inside out’ signalling (McDowall et al., J. Clin Invest. 111,
51-60, 2003). The LAD-III lesion has been attributed to a
C>A mutation in the CalDAG-GEF1 gene on chromosome
11q13.1 that codes for the protein. CalDAG-GEF1 is a good
LAD-III candidate protein as it activates the GTPase Rap-1
that is important for integrin activation. However Lena
Svensson, Alison McDowall and Irene Patzak in a collaboration
with Ian Tomlinson’s lab, have shown that this change is not
responsible for the LAD-III disorder. Instead we have identified
mutations in the nearby FERMT3 gene, that specifies the
Kindlin-3 protein, as the cause of the LAD-III disorder
mutations in the FERMT3 gene in Maltese and Turkish patients
result in decreased Kindlin-3 mRNA and loss of protein
expression. Importantly, transfection of the patients’
lymphocytes with Kindlin-3 cDNA, but not CalDAG-GEF1
restores adhesion and migration. There is evidence to suggest
that Kindlin-3 enables talin to bind to integrin, but much remains
to be discovered about how this new ‘player’ promotes the
activation of integrins on immune cells and platelets.

Leukocyte migration in vivo and in vitro
Neutrophils are the first immune cells to migrate into
infected tissue sites. It is therefore important to understand
how they are recruited. In an in vivo study in mice, Katia De
Filippo finds that resident tissue macrophages are the source of
the major neutrophil chemotactants, KC and MIP-2 (De
of these chemokines is rapidly regulated at the transcriptional
level by signalling through the toll-like receptors TLR2, TLR3
and TLR4 that have diverse specificities for pathogens. Thus
TLR signaling by tissue macrophages directly controls the
synthesis of neutrophil-attracting chemokines that are
essential for the earliest recruitment step in the innate
immune response to microbial challenge.

The S100 protein S100A8 is expressed by myeloid cells
and, unexpectedly, also by early embryos
We have been interested for some time in two small Ca2+
binding proteins, termed S100A8 (MRP-8) and S100A9
(MRP-14), which form a heterodimeric complex in myeloid
cells. Although these proteins are very abundant, making up
~40% of human neutrophil cytosolic protein, their function
has long been uncertain. We have performed targeted disruption
of both the S100A8 and S100A9 genes in mice. As the
S100A9−/− mice are viable (Hobbs et al., Mol. Cell. Biol. 23:
2564-2576, 2003), it was a surprise to find that S100A8 mice
are embryonic lethal indicating that S100A8 has a function
beyond heterodimer formation with S100A9. Jonathan Baker
and Meg Mathies find S100A8 is required prior to the
pre-implantation stage of embryo development and are
presently attempting to pinpoint where it is required in this
multi-step process. The hope is that an understanding of its
function in the well-described stages of embryo development
will shed light on its role in myeloid cells and highlight a new
function for proteins of this class in embryo development.

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The Developmental Genetics Laboratory examines the molecular processes that underlie animal development, in particular, the mechanisms used to generate the complex architecture of an organism. We are particularly interested in the process of segmentation — the formation of repeated units along the anteroposterior axis. Although developmental regulators and pathways have been highly conserved during evolution, the mechanism of segmentation differs considerably in the two systems we study — *Drosophila* and vertebrates. In *Drosophila*, segmentation occurs while the embryo is a single, multinucleate cell. In vertebrates and most other animals, segmentation occurs in a growing, multinucleate embryo, and requires neighbouring cells to signal between each other in order to coordinate their behaviours.

In *Drosophila*, we are studying how molecular motors contribute to one mechanisms for establishing organismal and tissue asymmetry: the localisation of mRNAs and other cargoes within cells. These studies provide insights into how the specificity of intracellular motors is determined, and how motor activity is regulated during patterning and in other developmental contexts. In vertebrates, we are analysing the circuitry and targets of the segmentation clock, the molecular oscillator that drives formation of successive intersegmental boundaries. We are also studying how stem/progenitor cells drive axial growth of the embryo.

DNA damage induces nuclear transport in *Drosophila* embryos

The first stages of *Drosophila* embryogenesis comprises 13 nuclear divisions that occur every 9–13 min, but in the absence of associated cytokinesis. This process generates a multinucleate syncytium, the single-celled blastoderm embryo, in which up to 6,000 nuclei are arranged as a layer around the cortex. Invaginating membranes then enclose each individual nucleus to form the somatic cells of the future larva and adult.

The rapidity of nuclear cleavage precludes most forms of DNA repair and checkpoint controls, and so the embryo has adopted a novel method of dealing with the consequences of mitotic disruptions. Damaged nuclei are excluded from the future organism by internalisation into the yolk region of the embryo.

We have been examining this form of organelle transport *in vivo* by following the motion of internalising nuclei labelled with fluorescent histones (Histone-GFP or –mRFP). We find that damage-induced internalisation is due to transport along microtubules (MTs): depolymerisation of MTs blocks internalisation, and inactivation of the blocking agent allows the resumption of transport (Figure 1a).

Because MTs are orientated in blastoderm embryos with their minus-ends apical, one might expect nuclear internalisation to involve plus-end-directed transport. Our
results indicate that this is indeed the case, and that internalisation is driven by the Kinesin-1 motor complex: timelapse imaging shows that internalisation is compromised in embryos mutant for components of this complex.

We are also investigating the signalling pathways that trigger internalisation. We have confirmed previous reports that DNA damage-induced internalisation is completely dependent on the activity of the chk2 check point gene, and are investigating (maternal) requirements for the upstream Rad9-Rad1-Hus1 (9-1-1) complex that is directly involved in sensing DNA damage. Internalisation is only delayed in hus1– embryos, implying that multiple pathways are involved in detecting DNA damage.

**lunatic fringe during segmentation**

During vertebrate segmentation, embryos generate bilateral pairs of new somites at regular intervals. In the mouse, 65 somite-pairs are generated, one every two hours. This repetitive process is controlled by the segmentation clock, whose action is revealed by oscillatory transcription of various genes in the presomatic mesoderm (PSM), the precursor tissue of the somites. The molecular composition of the clock is unclear although several pathways, including Notch, Wnt and Fgf signalling, have been implicated in regulating cyclic gene expression.

We have been analysing the role of lunatic fringe (lfng), a gene whose expression is activated by Notch signalling, and which encodes a potential inhibitor of Notch signalling. Lfng transcription occurs cyclically in the posterior PSM, and non-cyclically in a stripe at the anterior of the PSM where new somites are generated.

The relative importance of these two domains for segmentation is not known. Negative feedback of lfng activity could contribute to the segmentation clock in the posterior PSM. The anterior lfng stripe might have a role in generating the boundary that defines the new, forming somite.

To analyse the individual functions of each lfng domain, we made use of a promoter element that drives expression in only the anterior stripe domain. We generated transgenic mice in which this is the only lfng expression domain, i.e. that lack posterior cycling expression, and examined whether such selective lfng expression can rescue segmentation.

Unexpectedly, we find that requirements for oscillating lfng vary along the body axis. Anterior somites (numbers 1-30) require the cycling lfng activity for normal somite formation, but more posterior somites require only the striped expression. We also find that a small domain (somites 31-34) forms normal somites independent of lfng activity (Figure 1b). These results indicate that the circuitry defining somite boundary formation is modified during the course of axis elongation.

*Publications listed on page 123*
Membrane fusion is at the core of many essential cellular mechanisms including intracellular trafficking and mitotic organelle reconstitution. Without fusion, membrane traffic would cease and cellular compartments would shed vesicles until the compartments disappeared. To elucidate the mechanisms involved in membrane fusion, global lipid analysis of various membrane compartments is being performed and selective variation in species composition will be related to the physical properties that characterise membrane structure. Modulation of lipid species composition defines both the conformational changes of the membrane and the ‘local signal’. Perturbations caused by changes in membrane curvature and phospholipid composition affect the affinity of proteins to be targeted to appropriate membrane compartments.

In addition we are investigating how the regulation of membrane protein-lipid and protein-protein interactions, both inter- and intra-molecular, will be affected by the physical and compositional properties of the bilayer. Our main focus is to study and link, structural regulation and signalling during membrane fusion. Nano-analytical tools such as fluorescence lifetime imaging microscopy (FLIM) and other precise tools such as NMR spectroscopy and liquid chromatography tandem mass spectrometry (LC-MS/MS) are used to provide increased insight into molecular composition and associations in the cell and various sub-cellular compartments.

Proteo-lipid regulation in nuclear envelope assembly

Regulation of nuclear envelope dynamics is an important example of the universal phenomena of membrane fusion and fission. The nuclear envelope is disassembled and reassembled at each mitosis in typical animal cells. The processes of disassembly and reassembly may also occur in interphase and are usually but not always co-ordinate in nuclei sharing a common cytoplasm, for example in fertilised eggs. Male nuclear envelopes however are disassembled and reassembled in all cases. The study of the processes of male pronuclear membrane dynamics in fertilised sea urchin eggs investigated with cell free extracts has revealed several novel features, especially regarding the role of phospholipids during nuclear membrane formation. Our novel model combines and relates the study of membrane domains and regulation of membrane fusion. The fusion of chromatin-bound membrane vesicles is a process triggered by GTP, acting upstream of an endogenous PLC activity.

We have recently identified a sea urchin PLCγ isoform acting downstream of GTP. PLCγ is recruited to the nuclear envelope both in vitro and in vivo on vesicles (in collaboration with DL. Pocia, Amherst College, USA). The lipidome of these vesicles has been analysed with the HPLC-tandem mass spectrometer in Cell Biophysics and is rich in poly-phosphoinositides, including the PLCγ substrate PtdIns.
Our data reveal that PLC\(_4\), when activated in response to GTP, controls the fusion of nuclear envelope precursor membrane vesicles, by the generation of the fusigenic lipid diacylglycerol (DAG) from PtdIns(4,5)P\(_2\). We show the intersection of tyrosine kinase and phosphoinositide signalling pathways during membrane fusion. We have identified a sea urchin src family kinase (SFK) colocalised with PLC\(_4\) in vivo and in vitro. The site of this colocalisation is on MV1, a cortical vesicle population of unprecedented lipid composition, being 60% phosphoinositides (of which one-fifth is the PLC\(_4\) substrate PtdIns(4,5)P\(_2\)). MV1 is absolutely required for the formation of the nuclear envelope. Moreover, in collaboration with EJ Dufourc (CNRS-European Institute of Chemistry and Biology, (IECB) Bordeaux, France), solid-state nuclear magnetic resonance spectroscopy (ssNMR) was used to define the structure of the natural membrane domains in our model. Our future work in the regulation of NE assembly encompasses both somatic and non-somatic cells.

Protein kinase B (PKB/Akt) activation mechanism: from structure to function

The serine/threonine kinase PKB/Akt has received considerable attention over recent years and has become the focus of drug targeting for cancer therapy since a major role for this protein in tumour progression has emerged. We have tackled a very challenging problem of defining in vivo how a process works, leading to a different view of the molecular process.

Our results provide novel insights into the processes involved in the in vivo conformational change of the protein kinase and its association with PDK1, namely that it precomplexes in the cytoplasm with PDK1. The full-length structure of PKB/Akt has not been resolved and the key aim of our most recent investigation was to elucidate PKB/Akt three-dimensional structure in relation to its function.

By using a multi-disciplinary approach including molecular dynamic simulations, classical biochemical assays and FRET/two-photon FLIM, essential insights into the quaternary structure of PKB/Akt, in its inactive conformation was demonstrated. The use of molecular dynamic simulations challenged by mutagenesis experiments revealed a novel structure at the interface of the PH and kinase domains in the PKBa inactive conformation (a PH-domain induced cavity). The critical role of this novel structure in the regulation of the PKB inactive state was substantiated by its connection to the C-terminal regulatory domain of PKB: the hydrophobic motif.

These findings have led to an understanding of the molecular mechanism of action of a new class of highly selective allosteric PKB inhibitors. It elucidates at the molecular level its selectivity towards the different PKB/Akt isoforms that had remained so far elusive. We anticipate this will facilitate the optimisation of a new generation of more selective allosteric inhibitors.

Prognostic value of an activation state marker for EGFR and its downstream pathways in tissue microarrays of breast tumours

Current method for assessing patient’s entry for treatment of signal transduction inhibitor is limited. About 30% of breast cancer patients overexpress the ErbB2 receptor but of those patients, only 35% with 3+ over-expression respond to Herceptin. This indicates the limited capacity of the immunohistochemistry alone in predicting treatment success of these patients as the over expression of the ErbB2 may not correlate with the functional status of these receptors. A methodology to improve assessment of not just concentration of signal transduction target but their status is required. We have developed an innovative analytical approach to measure functional status of these signal transductions using FRET efficiency by high throughput fluorescence lifetime microscopy (FLIM) in tumour arrays. We have validated this FRET method to assess ErbB1 functional status in cells and head and neck tumour and various breast cancer cell lines. The FRET efficiency by FLIM has great potential to be a prognostic marker molecular therapy. Current work is being done on breast tumours in collaboration with Peter Parker’s laboratory (Protein phosphorylation laboratory). The samples are available through collaboration with Anthony Kong and Adrian Harris’s laboratory (Oxford-CRUK). The high throughput screening system has been implemented for tumour array analysis in collaboration with Pierre Leboucher (College de France-CNRS – Paris).

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The Notch pathway provides the most direct route for exchange of gene-regulatory signals between adjacent cells. It is critical for coordination of gene expression in almost all animal tissues. Feedback loops built from Notch pathway components drive cell diversification and generate pattern both in space and in time. To understand how tissues become organised during development, maintain their organisation during tissue renewal, and suffer disorganisation in cancer and other diseases, we need to understand the logic and dynamics of the Notch control circuitry, where and when it is brought into play, and what functions it performs in specific situations. We have continued to explore these questions in a variety of tissues, both experimentally and by mathematical modelling, using the zebrafish as our main experimental organism.

In birds, the array of auditory hair cells and supporting cells, once formed, is normally stable, with no cell turnover; but if hair cells are destroyed, the surviving supporting cells resume proliferation and serve as stem cells for regeneration of hair cells. In collaboration with the laboratories of N. Daudet (University College London) and J. Stone (Washington University, Seattle), we have investigated the role of Notch signalling in these switches of cell behaviour. The results show that Notch signalling is not required for maintenance of the quiescent state—which evidently depends on some other type of signal—but is reactivated during regeneration.

Gut

In the lining of the intestine, Notch signalling controls the differentiation of secretory and absorptive cells, again through lateral inhibition. In this system, however, new differentiated cells are continually being produced from stem cells, and the sequence of developmental decisions is more complex than in the ear. Our current work in the zebrafish indicates that Notch signalling is critical not only in controlling the basic choice between stem, secretory, and absorptive cell fates, but also in controlling choices between different secretory subtypes. From our conditional knock-out of the Delta1 gene in the mouse intestine, it seems that the same is true in mammals. But the details remain unclear for lack of knowledge of the precise lineage relationships of the different classes of cells in the gut lining. This has prompted
us to develop a new tool for clonal analysis of cell lineage and gene function in the zebrafish.

Cell lineage analysis

Clonal analysis hitherto has been difficult in vertebrates. Our new line of transgenics, TgMAZe, for Mosaic Analysis of gene function in the Zebrafish, should make the task easier. The transgenic construct incorporates a heat-shock promoter that drives expression of Cre. Upon induction, the Cre gene excises itself, and this brings a Gal4-VP16 construct under the control of a constitutive EF1a promoter. The construct also incorporates a UAS:mRFP gene, giving heritable expression of a nuclear-targeted mRFP marker in the clone of cells where the heat shock has triggered self-excision of Cre. By crossing TgMAZe fish with fish carrying an additional UAS-driven transgene or combination of transgenes, we can create marked clones of cells in which the Notch pathway is activated or blocked, either by itself or in combination with other pathways. We can then compare their behaviour with that of clones that simply express the RFP marker.

Our new methods of clonal analysis promise to have many applications, not only to tissues such as the lining of the gut and the inner ear, but also to cell populations such as the neural crest and vascular endothelial cells, whose migratory behaviour we have found to depend on Notch signalling.

Somite segmentation clock

Feedback loops in the regulation of genes in the Notch pathway underlie the remarkable temporal oscillations of gene expression that control the segmentation of the vertebrate body. In the zebrafish, our past work has led us to a quantitative theory of this ‘segmentation clock’, according to which the oscillations in individual cells arise from delayed negative feedback in the autoregulation of the Notch target genes her1 and her7, while neighbouring cells are kept in synchrony through communication via the Notch pathway. By blocking Notch signalling with DAPT, we have now shown that in this system Notch-mediated cell communication is needed only for synchronisation. We have been continuing our efforts to measure more of the key parameters and to clarify the role of certain other members of the her gene family that have been shown to oscillate in parallel with her1 and her7.

Each body segment corresponds to one tick of the segmentation clock, but what dictates the total number of segments? In a collaboration with the laboratory of O. Pourquié (Stowers Institute, Kansas City), we have compared four vertebrate species: zebrafish, chick, mouse, and corn snake. We find that the striking differences in segment number between chick, mouse, and snake mainly reflect differences in the rate of the segmentation clock relative to the rate of cell division in the presomitic mesoderm at the tail end of the body; in the zebrafish, however, the relatively small number of body segments is a reflection of a much briefer program of cell divisions in this growth zone and an earlier halting of the segmentation clock. These findings raise fundamental questions about the long-term programming of growth in the embryo – questions that are highly relevant to the problem of growth control in cancer.
The lymphatic vasculature constitutes an intricate network of vessels critical for fluid homeostasis, immune surveillance and fat absorption. In cancer, the metastatic tumour cells can exploit the lymphatic vasculature and spread via the lymphatic vessels to the lymph nodes. Our goals are to understand the cellular and molecular processes that form and maintain the lymphatic vessels and to use this knowledge to gain insights into the mechanisms underlying pathological conditions involving lymphatic vasculature, such as tumour metastasis and lymphoedema.

The lymphatic capillaries form a network of blind-ended vessels, which are distributed throughout the tissue spaces in almost all organs. These vessels collect the extravasated tissue fluid and drain via collecting vessels first into lymph nodes and then to larger lymphatic ducts, which connect to the venous system. Skeletal motion, arterial pulsation and respiration cause movements that generate pressure gradients required for the maintenance of fluid flow. In addition, smooth muscle cells around the collecting vessels cause contractions while luminal valves prevent backflow.

Studies using genetically modified mice have recently provided insight into the developmental processes and molecular mechanisms regulating lymphangiogenesis. Vascular endothelial growth factor-C (VEGF-C), acting through its receptor VEGFR-3, has been established as a key regulator of lymphatic endothelial cell proliferation and lymphatic vessel sprouting needed for the establishment of the embryonic lymphatic vascular network (Figure 1a). This primary vessel network is subsequently remodelled during late embryonic and early postnatal development. Our previous studies have shown that the remodelling processes involve expansion and transformation of a uniformly sized primary capillary plexus into a hierarchical vascular tree, composed of lymphatic capillaries and collecting vessels. This remodelling involves changes in vessel morphology, branching pattern and diameter, and requires the formation of new vessels via sprouting from the pre-existing vasculature, the specification of collecting vessels via recruitment of smooth muscle cells and the formation of luminal valves (Figure 1a). While significant progress has been made in understanding the developmental processes and genes that are controlling the initial development of embryonic lymphatic vessels, lymphatic vessel remodelling and maturation are only beginning to be understood. By using a combination of mouse genetics and in vitro studies of primary lymphatic endothelial cells we aim at addressing how the functional network of highly specialised lymphatic vessels is established.

Developing tools for targeting and visualising the lymphatic endothelium in vivo

Tissue-specific and inducible methods are necessary to allow functional studies specifically targeting the lymphatic endothelium, however, currently these studies are hampered by lack of suitable mouse models. We have therefore generated transgenic mice that allow gene deletion and/or overexpression specifically in lymphatic endothelial cells in vivo using the Cre/loxP recombination system. We are also generating transgenic mouse lines expressing fluorescent reporter proteins specifically in lymphatic endothelia. These mice will be used to visualise lymphatic vessels in vivo to gain insights into the processes of developmental as well as tumour-induced lymphangiogenesis and the entry of metastatic cells into the lymphatic vessels.

Collecting lymphatic vessels and lymphoedema

Lymphoedema is a progressive and lifelong condition characterised by gross swelling of the affected limb,
accompanied by fibrosis and susceptibility to infections. Currently no effective treatment for lymphoedema exists but the management of symptoms relies on remedial massage and restrictive bandaging. Inherited or genetic mutations, which compromise the development or function of the lymphatic vessels, may subsequently lead to lymphoedema. However, the vast majority of all lymphoedemas is due to damage to the collecting lymphatic vessels, most often caused by surgery or radiation therapy. A recent study, which used a mouse model of axillary lymph node dissection, suggested that growth factor-induced regeneration and maturation of lymphatic vessels might provide a basis for future therapy of lymphoedema (Tammela et al., Nature Med. 2007). However, before such strategies can be efficiently employed, a better understanding of the molecular regulation of collecting lymphatic vessel development and of the pathophysiological mechanisms involved is needed. 

Luminal valves present in the collecting vessels (Figure 1b) ensure unidirectional fluid flow and the functionality of the entire lymphatic vascular system, highlighted by the lack or insufficient function of lymphatic valves as one of the underlying causes of human lymphoedema. We found that one member of the integrin-family, integrin-α9, is predominantly expressed in the endothelial cells of the lymphatic valve. Interestingly, integrin-α9 deficient mice displayed specific defects in the formation of luminal valves, which resulted in retrograde lymphatic flow and impaired fluid transport. Together with ephrinB2 and FoxC2, which we (Makinen et al., Genes Dev. 2005) and others (Petrova et al., Nature Med. 2004) have previously shown to play an important functions in lymphatic remodelling, these three genes represent the only known regulators of lymphatic valve morphogenesis to-date. Future research will address the intracellular signalling mechanisms critical for lymphatic valve morphogenesis. Towards this aim we will study ephrinB2 and integrin-α9-mediated functions in lymphatic endothelial cells via identification and functional characterisation of molecular interactors and downstream signalling pathways.
The levels of many regulatory and misfolded proteins are controlled by the ubiquitin-proteasome system (UPS). Recruitment of protein substrates to the UPS machinery is frequently carried out by the multi-subunit E3 ubiquitin ligases, the largest group of which is the SCF family (Skp1-Cullin1-F-box). Within SCF E3 ligases, it is the F-box subunit that mediates the recognition of substrates for ubiquitination. Previous work with our collaborator Dr. Heike Laman (Cambridge University) identified the F-box protein, Fbxo7, as a putative oncoprotein that uniquely distinguishes between the G1 cdks (cyclin-dependent kinases) and specifically enhances the levels of D-cyclin/cdk6 complexes (EMBO J. 2005, 24: 3104-16). More recently, Fbxo7 has been suggested to undergo auto-ubiquitination and was found by linkage analysis to be mutated in Parkinsonian-Pyramidal Syndrome. To further characterise Fbxo7, we have used a combination of structural, biochemical and genetic approaches to identify and characterise regions involved in protein interaction as a means to identify potential substrates for SCFFbxo7. Both affinity purification and yeast-two hybrid experiments identified the proteasome inhibitor-31 (PI31), a regulatory subunit of the immunoproteasome, as a putative interaction partner for Fbxo7. We then uncovered an unexpected structural and evolutionary link between Fbxo7 and PI31 by using crystallographic and bioinformatic analysis of a dimerisation domain unique to both proteins (J.Biol.Chem. 2008 823:22325-35). The structure of this domain (FP domain) was subsequently used to guide the design of site-specific mutants defective in their ability to homo-dimerise and/or hetero-dimerise Fbxo7 and PI31. These mutants are being used to assess the functional consequences of ablating the Fbxo7-PI31 interaction in vivo. Although PI31 appears not to be a substrate for SCFFbxo7, we are currently exploring whether PI31 could modulate SCFFbxo7 function by antagonising Fbxo7 homo-dimerisation.

Macromolecular assemblies involving actin
Actin is a major component of the cytoskeleton of all eukaryotic cells and plays a fundamental role in a wide
variety of cellular processes including cell morphology and cell motility. Monomeric actin (G-actin) can polymerise to form helical actin filaments (F-actin), whose organisation contributes to cellular mechanical strength. A wide variety of proteins modulate the assembly, rearrangement and disassembly of F-actin including the mammalian enabled (Mena)/vasodilator-stimulated phosphoprotein (VASP) family. These tetrameric proteins alter the geometry of assembling F-actin filaments by antagonising capping proteins and bundling actin filaments. Last year, we and our collaborators in the Cell Motility Laboratory showed that Mena function is regulated by the tumor suppressor Tes. Tes binds the EVH1 domain of Mena through a non-canonical Lim-domain mediated interaction, thereby blocking a crucial protein binding site on Mena (Mol. Cell 2007 28, 1071–1082). We are continuing our collaboration with the Cell Motility Laboratory by investigating the molecular interactions made by Tes with other associated cytoskeletal protein partners including actin-related proteins.

Actin has unconventional functions outside of the cytoskeleton in processes such as gene transcription, chromatin remodelling and signal transduction. In one documented example relevant to transcriptional regulation, G-actin binds to the serum response factor co-activator MAL (also known as MRTF-A, myocardin-related transcription factor A). This interaction determines MAL subcellular localisation by preventing its nuclear accumulation as well as repressing transcriptional activation by the MAL-SRF complex. The G-actin binding site resides within the MAL amino-terminus which contains three copies of the RPEL motif (Arg-Pro-X-X-X-Glu-Leu, where X is any residue), quite distinct from other actin-binding proteins. Mutations at invariant positions within each RPEL motif have been shown to impair interaction with G-actin and de-repress MAL activity.

We are investigating the structural basis for MAL:G-actin interaction in close collaboration with the Gene Transcription Laboratory. This year we reported the crystal structures of two different 32-residue RPEL peptides from MAL (RPEL1MAL and RPEL2MAL) bound to G-actin:latrunculin B:ATP (EMBO J. 2008, 27:1-11). Each RPEL peptide presents two consecutive helices and a helical cap to bind the G-actin hydrophobic cleft and a ledge on subdomain 3, regions known to be involved in actin polymerisation (Figure 1a). Although many G-actin binding proteins engage the hydrophobic cleft of actin (Figure 1b), only the vitamin-D binding protein binds G-actin in an equivalent ‘cleft-and-ledge’ binding mode as MAL. Our RPEL1MAL:G-actin structures explain the sequence conservation defining the RPEL motif, including the invariant arginine that lies within the R-loop connecting both helices (Figure 1a). Characterisation of the RPEL1MAL:G-actin interaction with fluorescence anisotropy and cell reporter-based assays by our collaborators has further validated the significance of actin-binding residues within an RPEL motif for proper MAL localisation and regulation in vivo. Our future efforts will focus on the architecture of the RPEL domain (containing the three tandem RPEL motifs) bound to multiple G-actin molecules and how this assembly affects MAL function and localisation.

Figure 1. a) molecular interaction of RPEL2MAL peptide (green) with G-actin (white/grey surface). b) similarity between RPEL2MAL (green) and other actin-binding proteins (see box for colour codings for individual actin-binding proteins).

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Genome analyses in fission yeast

We are part of an international consortium to generate and analyse a genome wide set of gene deletions. As part of the validation of this set of deletions strains and to also develop methodologies for screening fission yeast mutants we have examined a subset of 2650 viable haploid deletion mutants in collaboration with a number of other laboratories.

This collection has been used to investigate the role of the DNA glycosylase MutY, which is highly conserved and found in both eukaryotes and prokaryotes. MutY has previously been shown to be involved in the repair of oxidative damage. We have shown that myh1 (MutY in fission yeast) also genetically interacts with a number of DNA repair genes particularly Rad1 and Rad9, which are involved in the DNA damage checkpoint. Double mutants of rad1 myh1 and rad9 myh1 have increased UV sensitivity. This work extended the known range of DNA damaging agents that require MutY for DNA repair (Jansson et al., 2008).

The deletion collection has also been used to screen for deletion mutants showing defective growth in different conditions. In one study the role of oxidative stress in fission yeast was investigated and it was found that a number of deletion mutants with defective mitochondrial function led to increased oxidative stress and a reduced life span. There were 51 deletion mutants sensitive to growth on both respiratory proficient medium and hydrogen peroxide containing fermentable media and 19 of these were defective in mitochondrial function. These mutants exhibit elevated levels of reactive oxygen species (ROS), which has been linked to reduced life span in other organisms and may explain why these fission yeast cells have reduced life span (Zuin et al., 2008). In a second study a screen for cadmium sensitive mutants identified 237 genes, including genes involved in sulphur metabolism, inorganic stress and the cell cycle. As well as identifying genes with previously known function we also identified genes of unknown function. These genes will help elucidate the process of cadmium tolerance in fission yeast and will also help to reduce the number of unknown genes which is currently about 18% of the total 4914 protein coding genes in fission yeast (Kennedy et al., 2008).

In budding yeast synthetic lethal interaction networks identified using synthetic gene arrays (SGA) have been used to define interactions that regulate essential gene functions. However the extent to which these interactions have been conserved in other organisms is not known. Double mutants of 222 genes including ones involved in DNA replication, DNA damage repair,
chromatin remodelling and intracellular transport and 2650 deletion mutants have been constructed to identified interacting genes. These data were then compared with interaction studies from a literature screen and with data from budding yeast. We found that ~29% of interactions are conserved between the two organisms (Dixon et al., 2008). A second, similar approach identified conserved networks using an epistasis mapping (Emap), which also analyses double mutant phenotypes. These data were then compared with data from an Emap of budding yeast. We found that functional modules are conserved between the two yeasts but that the wiring between them may be different between the two organisms (Roguev et al., 2008).

Cell cycle
The cell cycle in fission yeast is typically eukaryotic and has distinct G1, S, G2 and M phases. The genome is around 14Mb and is replicated bi-directionally from origins of replication dispersed throughout the 3 linear chromosomes. Previous studies have estimated that it takes around 20 minutes to replicate the whole genome and it has also been shown that although there are around 900 origins of replication only about one third are used in any one S phase. We have used a stochastic hybrid modelling approach based on genome wide data of origin of replication efficiency and position to study genome replication. We observed that because of the stochasticity of origin firing there are regions of the genome that would not be replicated within the estimated length of S phase using conventional methods. Modelling showed that increased fork rate or increasing the number of origins did not reduce S phase to the reported length. We propose that in fission yeast the length of S phase is longer that previously reported and extends in to the ‘G2’ phase of the cell cycle (Lygeros et al., 2008).

We have also carried out a genome-wide screen for new cell cycle mutants to identify a near complete set of genes required for the cell cycle (Figure 1). We are using these to carrying a haploinsufficiency screen for cells that delay entry into mitosis and to create temperature sensitive mutants for further study of new cell cycle genes that are conserved in humans and not previously characterised in any other organism. These genes will be important for identifying new human genes important for cell cycle progression.

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Figure 1. Wild type fission yeast cells and deletion mutant cells grown in rich medium and stained with DAPI to show the nucleus. (A) Wild type cells and (B) deletion mutant of SPAC17A3.05c which encodes a DNAJ protein and was not previously identified as a cell cycle gene. This mutant displays a number of nuclear defects including enlarged nuclei and mis-segregated nuclei (see red arrows). Both A and B are the same magnification.
Cell migration

Underlying the invasive properties of cancers are cellular functions associated with migratory behaviours. Emerging evidence indicates that there are multiple contributory factors influencing migration, particularly speed of movement and direction. Our continued work in this area has focused on three broad issues related to the PKC superfamily. Does migratory dependence on specific PKC family members reflect unique patterns of expression, function or regulatory ‘wiring’? How does the PKC superfamily influence the spatial constraints on signals involved in migratory behaviour? Can we map migratory signalling pathways through siRNA screening?

The question of PKC family redundancy would appear to have multiple answers. In a migratory model displaying dependence on atypical PKC (aPKC) isoforms, knock-down of either PKCζ or PKCι partially inhibits migration while the combination knock-down has a greater effect. For the PKN1,2,3 isoforms the effect of knock-down appears to reflect expression patterns such that where abundantly coexpressed, combination knock-down is required to suppress migration. However this is not simple redundancy as it appears that there are selective regulatory inputs peculiar to the particular cell. Protein arrays and substrate specificity screens have been exploited to generate a series of direct targets of PKNs that are candidates for mediating the PKN role(s) in migratory responses.

The nature of PKC family outputs is in part to control the subcellular distribution of signalling events. This has been investigated extensively in relation to PKCζ action in migration and in PKCε dependent HGF/cMet induced migration. For the aPKCs, they regulate the delivery of signals to the leading edge, in a monolayer wound-healing model. The extent to which these events account for aPKC action in this model is being probed with genetically encoded, drug-responsive tools. For HGF/cMet, we have shown previously that PKCε influences a related signal delivery process. We have now demonstrated that the PKCε control acting on the delivery of activated cMet to a perinuclear compartment
is instrumental in determining HGF-dependent activation and nuclear accumulation of STAT3, an event which is required for HGF-induced migration.

To further elucidate roles in these migratory responses, HGF-dependent monolayer migration and a distinctive transwell migratory model have been employed in siRNA library screens to identify relevant regulators. Validation of hits is ongoing. In parallel an siRNA screen to map a specific PKC-dependent pro-invasive property has been established.

Survival
PKCα is upregulated in multiple glioblastoma cell lines. To assess whether this is involved in the phenotype and how intervention might influence glioblastoma behaviour, we have compared the effects of PKCα knock-down with PKC(α) inhibition. Notably, loss of PKCα in U87MG cells was associated with induction of apoptosis. This exhibited a strong threshold effect requiring efficient knock-down to elicit this response. Consistent with a survival role for PKCα, expression of a kinase inactive, dominant negative PKCα also induced apoptosis. Interestingly, the requirement for PKCα is not corroborated by catalytic site directed inhibitors. It appears that PKCα has a scaffolding function which is activity independent and dominates behaviour in these glioblastoma cells.

The distinctions between siRNA knock-down, expression of a dominant negative kinase inactive allele and inhibition of PKCα are of some significance. One critical aspect of this is the altered conformation associated with the kinase inactive mutation of the conserved lysine in the ATP binding pocket. This mutant is not phosphorylated in the kinase domain and retains a non-functional conformation, unlike the inhibited WT-PKCα. The nature of these states, their inter-conversion and their inhibition are the subject of ongoing functional and structural work with Dr Neil McDonald (Structural Biology Laboratory) and the Development Laboratory (CRT).

Division
PKC isoforms have been implicated in controls acting at various steps in the cell cycle. We have recently demonstrated a requirement for PKCε in the completion of cytokinesis. Inactive forms of PKCε accumulate at the midbody and are associated with a slow down or complete failure of cytokinesis – the latter leading to binucleation, a prelude to aneuploidy (Figure 1). Analysis of the effectors located at the furrow alongside inhibited PKCε has identified RhoA. It appears that PKCε’s role in this final step is to regulate the switch off of RhoA, an event required for the disassembly of the contracted actin ring. The trigger(s) for recruitment of PKCε to the furrow, the dynamics of its association with the midbody and the proximal effector(s) responsible for RhoA control are the subject of ongoing work.

PKCε is engaged in this final irreversible step through a series of phosphorylations that enable the assembly of a complex with 14-3-3, which in turn confers constitutive activity on PKCε. The mapping of the docking sites has defined interaction within the V3 domain and the structure of the V3 domain/14-3-3 complex has been solved in collaboration with Dr Neil McDonald’s Laboratory (Structural Biology Laboratory).

Figure 1. Failed cytokinesis following induction of an inactive PKCε mutant. Cells (293T) harbouring an inducible, kinase inactive PKCε mutant were induced for 24h and then filmed. Shown is a series of phase images illustrating the progression of mitosis, through typical metaphase, anaphase and furrowing stages, but in this context ultimately leading to a failure of cytokinesis. The final image depicts the binucleate cell derived from this failed division. For these cells, 24h post-induction of the kinase inactive PKCε mutant the majority of cells are binucleate and a further 24h later most cells are dead.

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The \textit{INK4b-ARF-INK4a} locus encodes three proteins, each of which is capable of bringing cell proliferation to a halt. The two INK4 proteins, p16\textsuperscript{INK4a} and p15\textsuperscript{INK4b}, do so by blocking the phosphorylation of the retinoblastoma protein (pRb) by cyclin dependent kinases, whereas p14\textsuperscript{ARF}, so named because it exploits the second exon of \textit{INK4a} in an alternative translational reading frame, blocks the ubiquitin-mediated turnover of p53. The locus is kept transcriptionally silent in most normal cells in vivo but is activated by a variety of intracellular and extracellular cues, such as prolonged mitogenic stimulation, telomere erosion, oxidative stress or the presence of an activated oncogene. Explanting cells into tissue culture is enough to activate the locus, leading eventually to senescence. Whereas p16\textsuperscript{INK4a} seems to be the major determinant of senescence in human cells, p15\textsuperscript{INK4b} assumes this role in chicken cells, presumably because chickens have lost the capacity to encode p16\textsuperscript{INK4a}. In contrast, senescence in mouse cells is primarily dependent on the ARF/p53 pathway.

Regulation of \textit{INK4b-ARF-INK4a} by Polycomb group proteins

Current evidence suggests that in stem and early progenitor cells, the \textit{INK4b-ARF-INK4a} locus is transcriptionally repressed by the actions of the Polycomb group (PcG) of proteins. These proteins participate in at least two types of multi-component complex, termed Polycomb repressive complexes 1 and 2 (PRC1 and PRC2). PRC2 contains the histone methyl transferase EZH2 which catalyses trimethylation of histone H3 on lysine 27 (H3K27me3). This mark is recognised by the PRC1 complex which mono-ubiquitylates histone H2A on lysine 119, thereby shutting down transcription (Figure 1). Whereas the PRC1 complex in \textit{Drosophila} comprises stoichiometric amounts of four proteins, Pc, Psc, Ph and Sce, each has multiple orthologues in mammalian cells.

To try to define the PRC1 complexes that regulate \textit{INK4a}, we have used tandem affinity purification and mass spectrometry to identify proteins that co-purify with the Pc homologue CBX7, and the Psc homologues BMI1 and MEL18. Our analyses suggest that although there are multiple variants of PRC1 in human cells, each contains a single representative of the Pc, Psc, Ph and Sce families. Surprisingly, we find that multiple PRC1 components are associated with chromatin at the \textit{INK4a} locus and that shRNA-mediated knockdown of any one of these can result in loss of H3K27me3 at the locus and derepression of \textit{INK4a}. The most likely explanation, for which we are seeking additional evidence, is that \textit{INK4a} is regulated by a complex of PRC1 complexes.

Our research focuses on the regulation of the \textit{INK4b-ARF-INK4a} tumour suppressor locus and its role in the implementation of cellular senescence, the state of permanent growth arrest elicited by various forms of stress. As well as serving as a front line defence against oncogenic insults, senescence sets limits on the proliferative potential of stem and progenitor cell populations. The current priority is to determine how oncogenic signalling affects transcription of the locus by altering the state of histone modifications in the neighbouring chromatin.

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Proteins that co-purify with Polycomb complexes

As well as known PcG proteins, the mass spectrometry identified non-PcG components that may be implicated in PRC1 function. These include two ubiquitin proteases that might be involved in editing the mono-ubiquitylation of histone H2A by PRC1 or possibly in removing ubiquitin from H2B. This would serve to reinforce transcriptional repression by the complex. We also found an RNA helicase and are exploring the attractive idea that RNA interference or non-coding RNAs are required to establish PcG-mediated gene silencing. Importantly, knockdown of these non-PcG proteins with shRNA results in loss of PRC1 binding at the INK4a locus and p16INK4a-dependent senescence. We are using similar strategies to investigate protein kinases that consistently co-purify with PRC1 complexes, and in particular asking whether they phosphorylate histones or components of PRC1.

Reversal of PcG-mediated repression by oncogenic signalling

In collaboration with colleagues at the MRC Clinical Sciences Centre in London and Mount Sinai Medical Center in New York, we have been investigating how signalling from the RAS-RAF-MEK pathway impacts on PcG-mediated regulation of the INK4b-ARF-INK4a locus. Oncogenic RAS causes a reduction in H3K27me3 at the INK4a promoter by downregulating the EZH2 methyl transferase and upregulating one of the recently described H3K27me3 demethylases, JMJD3. In human fibroblasts, JMJD3 activates INK4a, but not ARF, and causes p16INK4a-dependent arrest. In contrast, in mouse embryo fibroblasts, JMJD3 activates INK4a and Arf and elicits a p53-dependent arrest, echoing the effects of RAS in this system. Our findings directly implicate JMJD3 in the regulation of INK4a/ARF during oncogene-induced senescence and suggest that JMJD3 has the capacity to act as a tumour suppressor.

Interestingly, the effects of JMJD3 do not extend to INK4b despite clear evidence that p15INK4b is also induced by RAS. In contrast, the MYC oncogene appears to have opposing effects on INK4b and INK4a. We are therefore conducting more detailed analyses of histone modifications (e.g. H3K27, H3K36, H3K4 etc) throughout the INK4b-ARF-INK4a locus and how they are altered in response to various agents and stresses. We are also extending these studies to chicken cells in which the PRC1 binding region of INK4a is missing.

Reciprocal regulation of INK4a and INK4c

In studying the role of the INK4 family in senescence, we noticed that when p16INK4a accumulates during replicative or oncogene induced senescence, the expression of p18INK4c is turned off. This occurs at the level of transcription and is not simply a case of one protein competing with the other for binding to CDKs. Curiously, much of the p18INK4c in proliferating fibroblasts is specifically associated with CDK6. As oncogenic RAS has reciprocal effects on p16INK4a and p18INK4c, we are extending the analyses of histone modifications to include INK4c.

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**Figure 1. Transcriptional repression by the Polycomb group proteins.** The PRC2 complex, comprising Ezh2, Eed and Su(z)12, establishes epigenetic marks in chromatin by tri-methylating histone H3 on lysine 27 (H3K27). This mark is recognised by the PRC1 complex, which mono-ubiquitylates histone H2A on lysine 119, leading to transcriptional repression. Whereas the PRC1 complex in Drosophila comprises stoichiometric amounts of Pc, Psc, Ph and Soe, there are multiple orthologues of these proteins in mammalian cells, as indicated in the coloured boxes.
From these tissues DC migrate into lymph nodes, spleen and other secondary lymphoid organs carrying antigens captured at the site of origin. Similarly, DC ordinarily resident in secondary lymphoid organs capture antigens that reach those tissues via the lymph or blood.

Depending on context, the presentation of antigens by DC can lead to T cell expansion and productive immune responses but can also inactivate T cells or promote the development of suppressor cells that maintain tolerance [Steinman et al., (2002). Proc. Natl. Acad. Sci. U.S.A. 99:351-358]. Therefore, the specific targeting of antigens to DC allows for numerous possibilities of immune manipulation, ranging from the induction of tolerance to improved vaccination against infectious diseases and cancer.

**DNGR-1**

To target antigens specifically to DC one needs to identify cell surface markers restricted to those cells. Such markers should act as endocytic receptors that will deliver bound cargo to compartments where antigen processing for MHC class I and class II presentation can take place. Several years ago, we had identified a fragment of a C-type lectin receptor in a screen designed to find transcripts selectively expressed in a subset of DC called CD8α+ DC. The full cDNA was subsequently cloned and turned out to correspond to the clec9a gene, part of the NK lectin gene family that also includes Dectin-1, another C-type lectin of interest to the lab. We subsequently confirmed the DC-restricted expression pattern of clec9a in mouse and human and, accordingly, named the protein product Dendritic cell NK lectin Group Receptor 1' (DNGR-1) [Sancho et al., (2008) J Clin Invest 118:2098-2110]. We made monoclonal antibodies to mouse and human DNGR-1 and used them to identify the cell populations that express the receptor. In the mouse, DNGR-1 is expressed at high levels by CD8α+ DC and at low levels by plasmacytoid DC (pDC) but not by other mouse hematopoietic cells [Sancho et al., (2008) J Clin Invest 118:2098-2110]. DNGR-1 is expressed at the plasma membrane but possesses endocytic activity and internalises bound antibody [Sancho et al., (2008) J Clin Invest 118:2098-2110]. This fact, together with its selective expression pattern, suggested that DNGR-1 might be useful for antigen targeting to DC, much like another C-type lectin known as DEC-205 [Hawiger et al., (2001) J. Exp. Med. 194:769-779, Bonifaz et al., (2002) J. Exp. Med. 196:1627-1638]. We went on to show that fluorescently-labelled anti-DNGR-1 mAbs injected into mice selectively label CD8α+ DC (strongly) and pDC (weakly) and that a derivative of the OVA 257-264 H-2Kb-restricted peptide covalently coupled to anti-mouse DNGR-1 is selectively crosspresented on MHC class I by CD8α+ DC in vivo [Sancho et al., (2008) J Clin Invest. 118:2098-2110]. When given with adjuvant, the same conjugates induce potent OVA-specific cytotoxic T cell responses, which can prevent development, as well as mediate eradication, of OVA-expressing B16 melanoma [Sancho et al., (2008) J Clin Invest 118:2098-2110]. However, to make the approach more relevant to cancer therapy, we also determined whether immunity could be achieved against relevant tumor antigens. This was achieved by conjugating anti-DNGR-1 to
peptides corresponding to endogenous melanocyte differentiation antigens that can act as B16 tumor rejection antigens in H-2b mice. Notably, when given therapeutically, a single dose of 2μg of conjugates (equivalent to 50ng of peptides), together with adjuvant, is able to break self-tolerance and induce crosspriming of CTL, which mediate eradication of growing tumour [Sancho et al., (2008) J Clin Invest 118:2098-2110]. Thus, targeting antigens to DNGR-1 is a promising approach for therapeutic vaccination, at least in the mouse. An exciting development is that we and others have found that human DNGR-1 is also highly restricted in expression to a small subset of blood DCs [Sancho et al., (2008) J Clin Invest 118:2098-2110, Caminschi et al., (2008) Blood 112:3264-3273, Huysamen et al., (2008) J. Biol. Chem. 283:16693-16701]. This suggests it might also be useful for selective DC targeting in humans and sets it apart from DEC-205, which, in human, has a broad distribution and is unsuitable as a target [Kato et al., (2006) Int Immunol 18:857-869]. We have filed a patent application on DNGR-1 as a novel and highly specific marker of mouse and human DC subsets that can be exploited for antigen delivery and for CTL crosspriming and tumor therapy.

**Dectin-1**

In order to ensure that the response to antigen culminates in immunity, antigen targeting to DC needs to be combined with additional strategies to ‘activate’ the cells [Reis e Sousa, C. (2006) Nat Rev Immunol 6:476-483]. Dectin-1, a phagocytic receptor for β-glucans expressed on yeast and bacterial cell walls (Figure 1), can function as a DC activating receptor [Rogers et al., (2005) Immunity 22:507-517]. Over the last year, we and others have shown that cell activation requires sustained signalling via Dectin-1, which takes place at the cell surface before ligand internalisation and is therefore especially prominent in response to ligands that are too large to be phagocytosed [Rosas et al., (2008) J. Immunol. 181:3549-3557, Hernanz-Falcón et al., (2009) Eur. J. Immunol. 29:507-513]. We further showed that Dectin-1-activated DC can induce the conversion of regulatory T cells into IL-17 producers [Osorio et al., (2008) Eur. J. Immunol. 38:3274-3281]. The latter observation extends our earlier studies on the induction of immunity dominated by IL-17-producing cells upon Dectin-1 triggering in vivo [Leibundgut-Landmann et al., (2007) Nat Immunol 8:630-638]. In addition, we have also shown that DC presenting OVA and stimulated with the Dectin-1-specific agonist, curdlan, are competent to prime OT-I TCR transgenic T cells in vitro and that curdlan serves as a potent adjuvant in vivo for the induction of OVA-specific cytotoxic T cells. The latter can protect mice from OVA-expressing B16 melanoma [Leibundgut-Landmann et al., (2008) Blood 112:4971-4980]. These studies indicate that the Dectin-1 pathway can also regulate CD8+ T cell immunity and could therefore be exploited in the design of cancer vaccines.
The goal of our work is to understand how cancer cells spread around the body. In order to address this problem we use a combination of in vivo tumour imaging, three-dimensional ‘organotypic’ models and conventional cell and molecular biology. Intravital imaging of tumours has revealed that only a subset of cells in primary tumours is motile and that this behaviour is not maintained at secondary sites. This could be because the activation of signalling pathways that promote cancer cell motility occurs locally and transiently. Furthermore, it has been suggested that motile cancer cells may become less differentiated or trans-differentiate.

To address these issues Silvia Giampieri and Sophie Pinner have developed strategies to monitor the activity of signalling pathways and the differentiation status of cells in both breast cancer and melanoma models. These two parallel projects have revealed an important role for transient activation of TGFbeta signalling. Together with Caroline Hill’s laboratory (LRI Developmental Signalling Laboratory) we are currently investigating the molecular mechanism by which TGFbeta signalling affects cell motility and whether it affects the differentiation status of the motile sub-population of cells. Following the departure of Silvia and Sophie in the autumn, Cerys Manning is continuing some of this work.

Our work using ‘organotypic’ models has previously shown the critical role of matrix remodelling by stromal fibroblasts in generating paths through the extra-cellular matrix that cancer cells subsequently use to invade (Figure 1). Cedric Gaggioli has now developed a 96 well assay for matrix remodelling by carcinoma-associated fibroblasts and screened a moderate size chemical library for compounds that prevent matrix remodelling. This approach identified several compounds that could prevent stromal fibroblasts from promoting the invasion of carcinoma cells without compromising the viability of either cell type. Steven Hooper has investigated the molecular mechanism by which one class of compounds identified in this screen reduces matrix remodelling. He has found that HMG-CoA reductase inhibitors reduce the membrane targeting of Rab family small GTPases and has identified specific Rab proteins that are required in fibroblasts to promote matrix remodelling but seem dispensible in carcinoma cells. One of these is critical for the delivery of integrins to the plasma membrane and subsequent interactions between the fibroblast and matrix.

Cristina Hidalgo and Shahid Chaudhry have been searching for genes involved in the fibroblast-led collective invasion of carcinoma cells. Cristina has demonstrated a role for genes involved in control of cell polarity in the collective invasion of carcinoma cells. Many of these genes were first identified in developmental biology systems and Chris Madsen, who joined the lab in September, will further investigate the role of genes involved epithelial morphogenesis in Drosophila in the invasion of epithelial cancers (collaboration with Barry Thompson, LRI Epithelial Biology Laboratory). SiRNA screening of regulators of ubiquitination by Shahid Chaudhry has led to a focus on the role of NFkB signalling and inflammatory cytokines in fibroblast-led invasion. Shahid is currently investigating the molecular mechanism by which regulators of NFkB signalling affect squamous cell carcinoma invasion.

In addition to the collective invasion of cancer cells our group is also interested in the molecular mechanics of how...
single cancer cells move around. Sophie Pinner has identified a novel role for PDK1 in this process (see Research Highlights) and has collaborated with Chris Marshall’s group at the Institute for Cancer Research to study single cell motility \textit{in vivo}. This work identified some of the control network that determines whether cancer cells move with an elongated morphology or a rounded morphology. Critically, the morphology or ‘mode’ of invasion affects whether cells are sensitive to potential anti-invasion drugs such as the ROCK inhibitor Y27632. We are now hoping to model aspects of these changes in cell morphology and mode of migration in collaboration with Paul Bates (LRI Biomolecular Modelling Laboratory).

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The goal of our laboratory is to build up a functional map of the organelles responsible for the endocytic sorting and axonal transport of neurotrophin receptors and selected virulence factors in neurons. By studying the basic mechanisms controlling neuronal membrane dynamics, we aim to provide new insights into how neurons control the uptake and sorting of ligands in health and disease.

An RNAi screen using neuronally-differentiated embryonic stem cells

The crucial role of axonal transport in cell homeostasis is underscored by the finding that mutations in genes encoding components of this machinery, such as motor complexes, cause neuronal death. Work from our laboratory has established that an atoxic fragment of tetanus toxin (H₇), which is transported in axonal carriers together with neurotrophins and their receptors, is a useful probe to monitor the dynamics of these complexes in neurons. The uptake of H₇ occurs via a specialised clathrin-dependent pathway involving a sorting step in which lipid microdomain-associated markers, such as polysialogangliosides, are retained on the plasma membrane. This route is thus a concerted microdomain- and clathrin-dependent endocytosis, two pathways usually viewed as mutually exclusive. Although our studies shed light on the role of the GTPase dynamin and clathrin adaptors in this process, our strategy has been largely based on low-throughput assays. A major obstacle in the implementation of unbiased strategies for gene discovery, such as high-throughput siRNA screenings, to the dissection of this process, is the paucity of cell lines that can be used as valid alternatives to primary neurons. Major advances in the field of embryonic stem (ES) biology have recently lifted this shortcoming by providing protocols for the differentiation of ES cells into specific neuronal subpopulations. We have optimised methods for the differentiation of an ES cell line expressing GFP under the control of a motor neuron (MN) promoter and the isolation of large amounts of ES-derived MNs. These cells are indistinguishable from primary MNs in terms of the expression of specific markers (Figure 1), ligand-specific endocytosis or axonal transport. These features make them a valid alternative to primary neurons and a unique resource for high-throughput screens using siRNA-based approaches. Using a library of siRNA pools targeting genes involved in endocytosis and membrane traffic, and a high-
throughput transfection method, we have performed a screen based on the endocytosis of a fluorescently-labelled HC and an antibody directed against the extracellular domain of the neurotrophin receptor p75NTR. High content image analysis has been optimised to detect the uptake of the ligands and to identify primary hits that have been subsequently re-screened using independent siRNA pools. Validated genes have been selected for further analyses to investigate the phenotype observed by RNAi. These results will greatly contribute towards building a road map of neurotrophin receptor endocytosis in primary neurons, providing at the same time insights into how a single receptor is able to mediate different signals leading to distinct physiological outputs.

**Distinct axonal transport pathways target pathogens and vectors for gene therapy to the central nervous system**

Pathogens exploit anterograde and retrograde transport pathways to enter and exit the central nervous system. In addition to the aforementioned studies using the H₂ fragment of tetanus toxin, we have recently characterised the internalisation and axonal transport of canine adenovirus serotype 2 (CAV2) and poliovirus, both of which preferentially infects neurons in vitro and in vivo.

Adenoviruses are widespread human pathogens and have been associated with brain tumours and severe CNS infections. Their relevance in human health and their potential use as vectors for gene therapy makes the understanding of the neuronal trafficking of these viruses very important. Major advantages of CAV2 vectors are their selective MN targeting when injected intramuscularly, and their long-lasting transgene expression in vivo. In addition to their potential for addressing fundamental questions in neurobiology, these features make CAV2-based vectors an ideal choice to treat CNS pathologies. In spite of their neurotropism, the trafficking of CAV2 has only been studied in epithelial cells or fibroblasts. We have recently filled this gap, exploring the determinants for CAV2 sorting and transport in motor and sensory neurons. CAV2 binding to axons was strictly dependent on the presence of its receptor CAR. Using fluorescently-labelled CAV2, we found that the virus moves bidirectionally in axons, with a bias for the retrograde direction. In contrast to the targeting observed in fibroblasts, the majority of CAV2 transport occurs in Rab7-positive compartments that display a neutral pH. CAR was found associated with these axonal organelles, which also contain other cargoes as diverse as HC and neurotrophin receptors. These results suggest that a single axonal carrier is capable of transporting cargoes targeting different membrane compartments in the soma. Moreover, our data suggest that CAR is a component of axonal carriers and that it promotes the entry of CAV2 into this compartment.

In an independent investigation, we also examined the trafficking of human poliovirus (PV) in neurons expressing its human receptor hPVR. Retrograde axonal transport is required for PV dissemination through the sciatic nerve of mice expressing hPVR, and for the appearance of a paralytic syndrome recapitulating the landmarks of the human poliomyelitis. Surprisingly, the axonal transport of PV was not completely abolished in the absence of hPVR, indicating that several different pathways for PV endocytosis axonal transport exist in vivo and in vitro. Altogether, our data demonstrates that distinct receptor-mediated endocytic events determine the sorting of diverse cargoes to non-degradative organelles, which are then recruited to long-range retrograde transport routes, in a process that allows endogenous ligands, pathogens and virulence factors to reach the CNS.

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Background
Akt kinases get activated in response to growth factors, cytokines and other growth promoting stimuli and are involved in the regulation of a number of cellular processes including metabolism, cell growth, proliferation and survival. Akt targets are involved in a number of cellular processes making this kinase a central component of many signalling pathways. This makes the Akt kinase a central component of many signalling pathways.

Some of the best-characterised Akt substrates are members of the FOXO family of transcription factors. Phosphorylation of FOXO proteins by Akt creates a binding site for 14-3-3 proteins causing cytoplasmic retention of the protein and results in inhibition of FOXO target genes. FOXO factors regulate the expression of genes involved in cell cycle regulation, de-toxification and stress response and the FOXO ortholog daf-16 regulates longevity in C. elegans; indeed, it has recently been confirmed that FOXO factors function as tumour suppressors in mice. Another major signalling pathway downstream of Akt involves the mammalian target of rapamycin complex 1 (mTORC1). mTORC1 regulates the activity of 56-kinases and 4-EBP which are involved in the regulation of protein translation.

Several oncogenic signalling pathways increase the glycolytic rate and induce a switch to aerobic glycolysis as the major energy source in cancer cells. Inhibition of oxidative phosphorylation provides cancer cells with a growth advantage under hypoxic conditions and aerobic glycolysis ensures energy production in cells with defective mitochondria, a feature of many cancer cells. However, cancer cells also need to adapt their metabolism in order to support the increased rate of macromolecule synthesis for cell growth and proliferation. Increased nutrient uptake and metabolism as well as anapleurosis are required to fulfil this metabolic demand. Importantly, specific alterations in metabolic activity found in cancer cells also provide novel targets for therapeutic intervention.

The role of lipid biosynthesis in the regulation of cell growth by Protein Kinase B (PKB/Akt)
Cell growth (accumulation of mass) needs to be coordinated with the metabolic processes that are required for the synthesis of macromolecules such as DNA, proteins and lipids. The PI3-kinase/Akt signalling pathway induces cell growth via activation of complex 1 of the target of rapamycin (TORC1). While the significance of mTORC1 dependent induction of protein biosynthesis for cell growth is well established, the contribution of lipogenesis is less well understood.

We have previously shown that Akt-dependent activation of the two rate-limiting enzymes of the fatty acid and cholesterol biosynthetic pathways, fatty acid synthase (FASN) and HMG-CoA synthase, requires SREBP and that Akt induces nuclear accumulation of mature SREBP1 (Porstmann et al., Oncogene 2005). We were subsequently able to show...
that Akt induces rapid accumulation of mature SREBP1 in the nucleus. Activation of SREBP by Akt was blocked by the mTORC1 inhibitor rapamycin and by ablating mTOR expression using siRNA. We could also show that silencing of SREBP attenuates Akt-dependent lipogenesis and limits the increase in cell size in response to Akt activation in RPE cells in vitro. More importantly, in vivo silencing of dSREBP in flies caused a reduction in cell and organ size (Figure 1) and activation of dSREBP was required for the induction of cell growth by dPI3K (Porstmann et al., Cell Metabolism 2008). These findings place the SREBP transcription factor downstream of a pathway that integrates growth factor signalling and nutrient availability and suggest that the PI3K/Akt/TOR pathway regulates protein and lipid biosynthesis in an orchestrated manner during cell growth.

Regulation of gene expression by FOXO3a
We have analysed the transcriptional programme induced by activation of FOXO3a in a colon cancer cell line (DLD1). Among the genes induced by FOXO3a were members of the Myc/Max/Mxd network of transcriptional regulators. We could show that FOXO3a induces expression of all four Mad/Mxd proteins, Mad1, Mxi1, Mad3 and Mad4. However, only Mxi1 was found to be a direct target of FOXO3a. Interestingly, induction of Mxi1 by FOXO3a is specific to the SRα isoform and is mediated by a cluster of highly conserved DBEs within the first intron of the mxi1 gene. We were also able to show that Mxi1 was required for efficient repression of a number of Myc target genes by FOXO3a. Furthermore, we observed that FOXO3a activation caused a switch in promoter occupancy from Myc to Mxi1 containing complexes on two E-Box containing regions of two Myc target genes. Silencing of Mxi1 and other Mad/Mxd family members reduced the cell cycle arrest and growth inhibition in response to FOXO3a activation (Delpuech et al., MCB 2007). These results demonstrate that the PI3-kinase/Akt/FOXO pathway can modulate Myc function, which could contribute to cell transformation and tumour development.

Identification of metabolic enzymes involved in glucose metabolism and lipid synthesis required for Akt-mediated carcinogenesis
Many cancer cells rely on glycolysis as a main source of ATP production even under conditions in which oxygen is not limiting. This phenotype, termed ‘aerobic glycolysis’ or ‘Warburg effect’ has been identified as a feature of cancer cells. Increased glycolysis increases hypoxia tolerance of tumour cells and provides cells with metabolites for the biosynthesis of macromolecules. Many tumours show increased expression of SREBP or fatty acid synthase (FASN) and exhibit increased levels of fatty acid synthesis. Furthermore, inhibitors to FASN show selective toxicity towards cancer cells. However, the role of FASN in cancer development is not yet fully understood.

In order to gain additional insight into the role of glucose and lipid metabolism in growth and survival of cancer cells, we have generated a collection of siRNA molecules that individually target 224 metabolic enzymes involved in glucose metabolism (glycolysis, TCA cycle, pentose phosphate pathway, fatty acid and cholesterol biosynthesis). We have chosen three prostate cancer cell lines, PC3, LnCAP and DU-145 and two immortalised prostate epithelial cell lines (PrEC-LH and RWPE-1) for our initial studies. We have identified a number of interesting enzymes that are selectively required for the survival of the three prostate cancer cell lines. Further studies are required to identify the contribution of these enzymes to cancer cell survival in vitro and in vivo.

Figure 1. Inactivation of dSREBP reduces wing size in Drosophila melanogaster. Wing area analysis of control flies and flies expressing dSREBP RNAi or dominant negative mutants of dSREBP in different compartments of the wing.

a) Inactivation of dSREBP in the dorsal cell layer of the wing causes a reduction in wing area. b) Silencing of dSREBP in the posterior compartment of the wing causes a selective reduction of this compartment.
During the process of cell division, it is essential that daughter cells receive the correct complement of chromosomes. An incorrect number of chromosomes (aneuploidy) is associated with varying pathological outcomes. Tumour development has been strongly linked to aneuploidy, while many genetic diseases such as Down’s syndrome are caused by loss or gain of single chromosomes. To prevent these conditions, cells have developed a sophisticated molecular machine which as well as segregating chromosomes during mitosis, has a surveillance role to ensure correct distribution.

Our laboratory is trying to understand the mechanism of this machine at a molecular level. In particular, we are interested in the kinetochore, a large multi-protein complex that attaches the condensed chromatids to the mitotic spindle, and provides the origin for the checkpoint signal. Many kinetochore proteins are subject to very rapid evolution, so it is often difficult to identify clear sequence motifs that may enable prediction of function, or to identify orthologs in different species. By using a combination of structural biology and in vitro biochemistry, we hope to clarify the functions of individual components of the kinetochore, and build up a model for the overall assembly.

Locating the centromere

In budding yeast, the chromosomal location of each centromere is determined by a tripartite DNA sequence motif, that contains an invariant binding site (CDEIII) for the Cep3 protein. This is a constituent of the CBF3 complex, a 480 kDa hetero-hexamer which is necessary for localisation and assembly of the rest of the kinetochore. Current evidence suggests that the purpose of CBF3 is to locate a centromeric nucleosome containing the histone H3 variant, CenH3 to the proximal CDEII sequence element. In addition to sequence-specific binding by Cep3, CBF3 contains the Ndc10 protein, which also binds the centromere in a CBF3-independent manner, and is required for completion of cytokinesis. Despite the importance of these proteins in multiple aspects of cell division, little is known about their structure or mechanisms. We have determined the structure of the Cep3 protein, and are now turning our attention to how the intact CBF3 complex is formed, and binds centromeric DNA. This has been complicated by the difficulty in producing sufficient quantities of recombinant protein. In collaboration with the Protein Production Facility at the LRI, we have overcome these problems, and currently re-constituting the intact CBF3 complex for further study.

The chromatin connection

It seems likely that the main mechanical connection to chromosomes occurs through sections of chromatin containing modified nucleosomes containing the histone H3 variant, CenH3. A large number of inner kinetochore proteins associated with centromeric chromatin have been identified; but it is not currently known which of these are responsible for directly binding the nucleosomes. In vertebrates, it is thought that the so-called CCAN proteins (constitutive centromere-associated network) contain multiple chromatin-binding activities that bind both CenH3 and conventional nucleosomes. Currently, about 17 distinct proteins have been implicated in the CCAN. The roles of the individual proteins, assembly hierarchy and relationships between them are unclear and somewhat controversial. Without such data, it has proved extremely difficult to carry out in vitro experiments on the proteins. However, several orthologs of the CCAN proteins have been identified in the simpler budding yeast inner kinetochore, and we are now attempting to map their interactions with the centromeric nucleosome.
The core of the kinetochore

Recent studies from various labs have identified a conserved super-complex of proteins, which appears to represent a central scaffold that directly links the inner layers of the kinetochore to the microtubule plus-tips. This is the so-called KMN complex, after the constituent sub-complexes, KNL1 (Spc105 in budding yeast), Mtw1 and Ndc80. Our understanding of the function of this complex has substantially advanced with the demonstration that Ndc80 can directly bind microtubules and forms a stable complex with Mtw1 and KNL1, an interaction that enhances the microtubule affinity (Figure 1a). Exactly what the functions of Mtw1 and KNL1 are is unclear. KNL1 alone has a weak affinity for microtubules but there are no obvious sequence motifs that can be directly related to this activity. The Mtw1 complex is equally mysterious. It has no intrinsic MT affinity, but has been suggested that it acts as a scaffold, to anchor the KMN complex and correctly orient the Ndc80 and KNL1 proteins for MT attachment. Importantly, all three sub-complexes are targets of the Ipl1/Aurora B kinase that is essential for the recognition and removal of incorrect MT-chromosome attachments. It is probable that phosphorylation of the Ndc80 MT-binding domains directly reduces affinity for tubulin subunits, but the function of the phosphorylation in the other proteins is unknown. We have successfully expressed intact, full-length versions of both the Ndc80 and Mtw1 complexes. The recombinant complexes can interact, and may be isolated as a single stable species (Figure 1b).

We are now attempting to reconstitute the entire complex including KNL1 for structural and biochemical analysis.

Figure 1. a) Schematic representation of the KMN complex. The coiled-coil containing Ndc80 complex can directly bind to microtubules via EB1-like domains (light and dark blue). KNL1 can also bind to MTs, and the Mtw1 complex appears to act as a scaffold, and contact the inner kinetochore. b) The Mtw1 and Ndc80 tetramers associate to form a 310 kDa octameric complex that may be purified as a discrete species in large quantities. The Coomassie-stained gel shows all eight individual proteins.

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Chemotherapy drug resistance in solid tumours results in treatment failure and the rapid deterioration in patient quality of life. Tailoring cytotoxic chemotherapy schedules to those patients most likely to benefit based on tumour molecular phenotype and the identification of patients who should be considered for clinical trials with novel therapeutic agents due to intrinsically drug resistant disease, may help to improve patient outcome and maximise the survival benefit associated with chemotherapy schedules. Our work is focused on identifying drug resistance pathways to common cytotoxics and targeted therapies used in medical oncology practice in order to support clinical strategies directed towards the more effective delivery of these agents (Swanton et al., (2008) Breast Cancer Res 10,214).

Quantification and relevance of CIN to microtubule stabiliser resistance in solid tumours.

Functional genomic screening data from our laboratory has revealed several genes that simultaneously regulate chromosomal instability (CIN) and response to microtubule stabilising agents such as paclitaxel (Swanton et al., 2007 Cancer Cell 11, 498-512). We are currently exploring the frequency of numerical chromosomal instability in primary breast cancer in collaboration with Dr Rebecca Roylance (Institute of Cancer) and the TRANS-TACT consortium. The consortium is analysing tumour samples from the phase III TACT clinical trial where patients were randomised to treatment with a taxane/anthracycline schedule or standard anthracycline based chemotherapy alone. We are investigating the expression of proteins known to be involved in CIN in these tumours and the relationship of CIN to clinical outcome following taxane therapy.

CINATRA (Chromosomal INstability and Anti-Tubulin Response Assessment) is a phase II clinical trial deriving from our laboratory programme that has recently opened at the Royal Marsden Hospital. We are investigating whether near diploid colorectal cancers display intrinsic sensitivity to the microtubule stabilising agent, Epothilone 906 compared to colorectal cancers with CIN.

Relevance of the multi-drug resistance protein CERT to drug resistance and clinical outcome.

The response rates to successive ‘non-cross resistant’ chemotherapy schedules decline as the cancer advances and lines of treatment progress. Expression of the ABC family of multi-drug efflux transporters does not fully explain the clinical phenomenon of multi-drug resistance in vivo. Through an RNA interference drug sensitivity screen we have identified a ceramide transporter, CERT, the silencing of which results in sensitisation to multiple cytotoxics (Swanton et al., 2007a). We have found that CERT is over-expressed in drug resistant cell lines and cytotoxic resistance can be attenuated by CERT depletion in these cells. Ceramide, termed a ‘pro-apoptotic lipid’, is generated through de novo synthesis or sphingomyelin hydrolysis. Ionising radiation and cancer cytotoxics induce many of the enzymes required for ceramide synthesis resulting in cell death through diverse mechanisms including the inactivation of AKT. In keeping with the importance of the ceramide pathway in the regulation of drug response, several enzymes in the ceramide biosynthetic pathway have been proposed to play a role in drug resistance both in vitro and in vivo (Figure 1).
We are attempting to understand how disruption of ceramide transport to the golgi apparatus promotes cell death following exposure to chemotherapeutic agents and the relevance of CERT expression to cytotoxic sensitivity in breast cancer. We have found that depletion of CERT in cells exposed to paclitaxel results in the induction of endoplasmic reticulum (ER) stress as measured by an increase in phosphorylated PERK. We are investigating the role of the ER stress pathway and ceramide generation within the endoplasmic reticulum in the initiation of cell death following cytotoxic chemotherapy exposure. Work in collaboration with Cancer Research Technology is investigating whether CERT could present a novel drug target to optimise drug sensitivity in cancer.

**Genome instability and drug resistance**

Patterns of genomic instability contribute to chemotherapy resistance and represent a common feature of malignancy. Aneuploid tumour cell lines that have acquired resistance to one chemotherapy agent have been shown to demonstrate resistance to non-cross resistant cytotoxic agents. This has led to the hypothesis that tumour aneuploidy may catalyse drug resistance through spontaneous chromosome reassortments, such that tumour cell selection for drug resistance to one chemotherapy agent is often associated with resistance to multiple unselected chemotherapy agents. We have shown that multi-drug resistance is a more frequent phenotype associated with gene silencing than resistance to single cytotoxics (Swanton et al., (2007) Cell Cycle 6, 2001-2004). This implies that non-cross resistant cytotoxic agents depend on the activation of similar cell survival pathways to promote drug resistance and may further explain the relative ease through which patterns of genomic instability may initiate resistance to common cytotoxic agents. We are using bioinformatics and whole genome functional approaches to attempt to identify common survival pathways in aneuploid tumour cells that may enable the more efficient and specific targeting of tumours to limit the rapid acquisition of drug resistance in vivo.

**Cytotoxics Enhance Production of the Pro-apoptotic lipid Ceramide**

Figure 1. Activation of enzymes required ceramide synthesis occurs following cancer cytotoxic exposure (blue). Proteins implicated in drug resistance are highlighted in red. The lipid transporter CERT and beta-glucosidase were identified by our laboratory as novel drug resistance molecules.
The Hippo (Hpo) pathway comprises the kinases Hpo and Warts, the adaptors Salvador and Mats, the cytoskeletal proteins Expanded and Merlin, the atypical cadherin Fat and the transcriptional co-factor Yorkie [Harvey et al., (2007) Nat Rev Cancer 7, 182-191]. This pathway has been shown to restrict tissue size through the control of cell division and apoptosis during development in Drosophila. The immediate aim of the lab is to identify new members of the Hpo pathway and to understand how the pathway functions at the biochemical level. The lab uses a combination of genetics, cell biology and biochemistry in order to understand how the Hpo pathway fits in the ‘big picture’ of overall size regulation in development and cancer.

The Hippo pathway and cell polarity
In addition to their well-characterised overproliferation phenotype, epithelial cells mutant for the kinases Hippo and Warts present a hypertrophy of the apical domain. We have examined the molecular basis of this apical hypertrophy and its impact on cell proliferation. In the wing imaginal disc epithelium, we observe increased staining for the apical polarity complexes, such as DaPKC/Par3/Par6 and Crumbs/Stardust when Hippo activity is compromised, while baso-lateral markers are not affected. The cell surface localisation of the Notch receptor is also increased in mutant clones, opening the possibility that aberrant receptor signalling may participate in overgrowth of hpo-deficient tissue. Interestingly however, while the polarity determinant Crumbs is required for the accumulation of apical proteins, this does not appear to significantly contribute to the overproliferation defect elicited by loss of Hippo signalling. Therefore, Hippo signalling controls polarity and growth via distinct mechanisms.

Disruption of epithelial architecture and loss of cell polarity is a hallmark of cancer. In breast or colon cancer, loss of polarised architecture is usually the first sign of transformation [Wodarz et al., (2007) Nat Cell Biol 9, 1016-1024]. Can loss of polarity therefore lead to cancer? Recent work on the neoplastic tumour suppressor genes (nTSGs) in Drosophila has put the link between polarity abnormalities and tumour formation into sharp focus [Harisharan et al., (2006) Annu Rev Genet 40, 335-361]. nTSGs such as the basal determinants scrib, dlg and lgl present a strong expansion of their apical domain, with ectopic localisation of apical proteins on lateral membrane and ectopic formation of adherens junctions. Those cells also have a massive overproliferation defect; they do not cycle faster than...
wild-type cells but they do not respond to arrest cues and carry on dividing. However, several studies suggest that, as is the case for Hpo pathway mutants, the proliferative and polarity defects are separable. Thus, there may be more similarities between neoplastic TSGs and hyperplastic TSGs such as Hpo pathway members than previously thought.

**Drosophila MFAP1 is required for pre-mRNA processing and G2/M progression**

In a non-Hippo related project, we have characterised a novel component of the spliceosome and its function in cell cycle regulation. The mammalian spliceosome has mainly been studied using proteomics. The isolation and comparison of different splicing intermediates has revealed the dynamic association of more than 200 splicing factors with the spliceosome, relatively few of which have been studied in detail. Here, we report the characterisation of the Drosophila homologue of Microfibril-Associated Protein 1 (dMFAP1), a previously uncharacterised protein found in some human spliceosomal fractions [Jurica et al., (2003) Mol Cell 12, 5-14]. We showed that dMFAP1 binds directly to the Drosophila homologue of Prp38p (dPrp38), a tri-snRNP component, and is required for pre-mRNA processing [Andersen et al., (2008) J Biol Chem 283, 31256-31267]. dMFAP1, like dPrp38, is essential for viability, and our in vivo data show that cells with reduced levels of dMFAP1 or dPrp38 proliferate more slowly than normal cells and undergo apoptosis. Consistent with this, dsRNA-mediated depletion of dPrp38 or dMFAP1 causes cells to arrest in G2/M, and this is paralleled by a reduction in mRNA levels of the mitotic phosphatase string/cdc25. Interestingly dsRNA-mediated depletion of a wide range of core splicing factors elicits a similar phenotype, suggesting that the observed G2/M arrest might be a general consequence of interfering with spliceosome function.

![Figure 1. Inactivation of Hpo pathway members induces apical protein accumulation. Transverse sections of a wing imaginal disc. Apical is to the top. Cells mutant for wts (negative for GFP, green in C) present an increase in the apical marker DaPKC (A and blue in C) but not the lateral marker Dlg (B, and red in C).](image_url)
The Thompson Lab is interested in how tissues control their growth and form during development. We use the fruit fly *Drosophila* as a model system for investigating this problem. The size and shape of tissues is determined by the coordinated behaviour of individual cells during development. This coordination is achieved by intercellular signalling pathways that control the growth and division of cells. We are conducting an in vivo RNAi screen in the fly wing to identify new components of these pathways, with a view to gaining a more complete understanding of how signalling pathways control tissue growth and form.

**The Wnt pathway**

The Wnt pathway is a developmentally regulated pathway that controls tissue growth and patterning in the fly. The range over which the Wnt ligand Wingless spreads is crucial for determining the size of the fly wing. Neil Pearson has identified a large number of RNAi lines targeting novel genes that produce Wnt-related phenotypes when expressed in the fly wing. Neil is currently testing whether these RNAi lines interact genetically with Wingless in the fly eye. Lines that score positively in both the wing and eye will be selected for further analysis. Neil aims to generate null mutants in selected candidate genes and determine the molecular function of the gene product in Wnt signalling.

**The Hippo pathway**

The Hippo pathway is a new tumour suppressor pathway that controls the proliferation and apoptosis of cells. Together with Nic Tapon’s lab, we are interested in identifying new components of the this pathway and how it is regulated during development. Eliana Lucas has identified an RNAi line causing apoptosis of cells in the fly wing, a phenotype closely resembling gain of function of the Hippo pathway. This RNAi line targets a novel gene encoding a protein that binds to Hippo in a yeast-2-hybrid assay. Eliana has generated a mutant in this gene, which confirms the RNAi phenotype. Eliana is also currently characterising the interaction between these two proteins in co-immunoprecipitation assays and performing genetic epistasis analysis to determine whether this novel gene acts genetically upstream or downstream of Hippo.

Ruth Brain and Barry Thompson have identified a second novel Hippo pathway component that causes tissue overgrowth when knocked down by RNAi. The RNAi phenotype closely resembles RNAi of *hippo* in both the fly wing and eye. Alice Genevet in Nic Tapon’s lab has generated a mutant in this gene that confirms the RNAi phenotype. Alice has also found that the protein product of this gene binds to the Hippo pathway components Expanded and Merlin (homologues of the human NF2 tumour suppressor). Alice is currently exploring how these proteins bind, whether they are transcriptionally co-regulated, and whether they act synergistically as tumour suppressors.

**The Insulin/TOR pathway**

The Insulin/TOR pathway regulates cell growth in response to nutrition. Alterations in this pathway change the size of cells, as well as the size of tissues. Clara Sidor has identified an RNAi line causing an increase in both cell and tissue size. This phenotype resembles that of RNAi for negative regulators of the Insulin/TOR pathway such as the PTEN tumour suppressor. Clara is currently generating a mutant in this gene to confirm the RNAi phenotype and to allow a more detailed phenotypic characterisation.
Epithelial polarity and shape

Most of the adult fly develops from epithelial tissues. Epithelial cells are polarised in their apical-basal axis and adopt particular shapes that determine the morphology of the overall tissue. Epithelial organisation allows signals to control development along a single, two dimensional plane. Understanding epithelial polarity and shape is therefore essential to understanding the development of growth and form of tissues. Furthermore, most human tumours are epithelial in origin and progression to metastasis requires that cells escape from the epithelium. Georgina Fletcher has conducted an RNAi screen for defects in epithelial organisation in the ovarian follicle cell epithelium. Georgina has identified a number of RNAi lines that cause loss of epithelial polarity and has confirmed these phenotypes by generating mutants in these genes. Interestingly, several of these RNAi lines are known to form a single protein complex that controls endocytosis. Georgina is currently investigating the links between endocytosis and polarity in these cells.

Figure 1. An in vivo RNAi screen for signalling pathways controlling tissue growth. Mis-regulation of Wnt, Hippo, or Insulin/TOR signalling can lead to defects in tissue size. Each pathway has characteristic effects upon cell size or cell fate that enables their phenotypes to be distinguished. The phenotype of selected signalling components is shown at the top. A schematic diagram of each pathway is shown at the bottom, we have identified novel compounds in each of these pathways.

Figure 2. RNAi of Lgl in the developing Drosophila wing causes loss of epithelial polarity (bottom) and tumour formation (top). Wild-type is shown on the left. Lgl RNAi (Lgl-IR) is shown on the right.
Spatio-temporal regulation of Kinesin 8 Klp5 and Klp6

Kinesins were originally identified as motor proteins that use energy gained from ATP hydrolysis to power processive movement along microtubules. Kinesins consist of a large protein family and recently, it has become clear that several kinesin subfamilies (namely kinesin-8, -13 and -14) possess microtubule depolymerising activities and are, therefore, important regulators of microtubule dynamics. Fission yeast Klp5 and Klp6 belong to the kinesin-8 family. Klp5 and Klp6 are not essential for cell division, but deletion mutants exhibit hyper-stable microtubules with defects in chromosome congression and segregation. Most kinesins function as homodimers. Klp5 and Klp6 are, however, unique members of the kinesin-8 family, as they are the only members reported thus far that form a heterodimer, which is essential for Klp5/6 function.

We have investigated the importance of Klp5/6 dimerisation. We show that Klp5 and Klp6 are mutually dependent on each other for nuclear mitotic localisation. During interphase, these two molecules are exported to the cytoplasm. In sharp contrast, during mitosis, Klp5 and Klp6 remain in the nucleus, which requires the existence of each counterpart (Figure 1). Canonical nuclear localisation signal (NLS) is identified in the non-kinesin C-terminal regions. Intriguingly individual NLS mutants (NLSmut) exhibit loss-of-function phenotypes, suggesting that Klp5 and Klp6 enter the nucleus separately. Indeed whilst neither Klp5-NLSmut nor Klp6-NLSmut enters the nucleus, wild type Klp6 or Klp5 respectively does so with different kinetics. Remarkably chimera strains containing only the N-terminal Klp5 kinesin domains cannot disassemble interphase microtubules during mitosis, leading to the coexistence of cytoplasmic microtubules and nuclear spindles with massive chromosome mis-segregation. In this strain a marked reduction of microtubule dynamism, including microtubule catastrophe/rescue frequency, shrinkage rate and dynamicty is evident. These results prompt us to propose that Klp5 and Klp6 play a vital role in promoting microtubule...

We have previously shown that fission yeast homologues of TACC (Transforming Acidic Coiled Coil protein) and TOG (Tumour Overexpressing Gene), Alp7 and Alp14 respectively, form a complex, which shuttles between the cytoplasm and the nucleus (Sato and Toda, 2007, Nature, 44, 334-337), reminiscent of Klp5/6 localisation patterns. Importantly Alp7/14 and Klp5/6 regulate microtubule dynamics in an antagonistic but coordinated fashion as microtubule stabilising and depolymerising factors, respectively, throughout the cell cycle. This fact raises an interesting possibility that common regulatory mechanisms, involving the Ran-GTPase signaling pathway (a central player for nuclear transport and spindle microtubule assembly), exist to ensure proper spatiotemporal control of microtubule morphogenesis. Further elucidation of such molecular mechanisms would lead to a better understanding of microtubule-dependent cell morphogenesis, bipolar mitotic spindle formation and coordinated chromosome segregation.

Mitotic role of the Dam1/DASH complex

The kinetochore, a specialised proteinaceous structure on the centromeric DNA, must attach to spindle microtubules in a bipolar manner to ensure high fidelity sister chromatid segregation. Budding yeast Dam1 (or DASH) complex is an outer kinetochore complex that consists of 10 essential proteins, Ask1, Duo1, Dam1, Dad1-4, Hsk3, Spc34 and Spc19. This complex is postulated to play an important role in facilitating the attachment of the spindle microtubules to the kinetochore in metaphase, but how it regulates microtubule dynamics remains unknown. Unlike budding yeast, the fission yeast complex is non-essential, however it promotes bipolar microtubule attachment in conjunction with microtubule-depolymerising kinesin-B Klp5 and Klp6 (see above). We screened for dam1 temperature sensitive mutants in a klp5 null background and identified the dam1-A8 mutant. dam1-A8klp5 double mutant cells display massive chromosome missegregation with lagging chromosomes and monopolar attachment of sister chromatids to one SPB (Spindle Pole Body, fungi equivalent of the animal centrosome).

Unexpectedly contrary to a dam1-deletion mutant that is hypersensitive to microtubule-destabilising drugs, dam1-A8 is resistant and furthermore the temperature sensitivity of dam1-A8klp5 is rescued by addition of these drugs. This indicates that the hyper-stabilised rigidity of kinetochore-spindle mal-attachments is the primary cause of lethality (Figure 2). Our result shows that fine-tuning of Dam1 activity is essential for chromosome bi-orientation. (Griffiths et al., 2008, Biochem. Biophys. Res. Commun. 368, 670–676).

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Genetic susceptibility to cancer
The possibility that common diseases, including cancers, have a genetic basis has been known for many years. Studies of familial risks and heritability have supported this contention. One plausible model is that there exist common genetic variants with individually small effects on disease risk. However, the chances of developing a disease could be markedly increased if an individual inherited several of these variants. Common disease-causing variants could be detected by association studies, comparing allele frequencies in cases and controls.

A few years ago, we obtained special funding from CR-UK for a genome-wide association study (GWAS) using the Illumina Hap550 tagSNP array to identify common bowel cancer predisposition genes. A multi-stage design involving up to 10,000 cases and controls was used, in collaboration with Richard Houlston (Institute of Cancer Research) and Malcolm Dunlop (Edinburgh). We also assembled an international consortium (CRACAC) of 8 groups from Spain, Finland, UK, Germany, Czech Republic and Australia.

This work is ongoing, but has already yielded 10 loci that are associated with an increased risk of colorectal cancer: These can be summarised as

- rs6983267 (chr8q24.21), a locus also involved in prostate and ovarian cancer predisposition, and adjacent to independent loci for breast and prostate cancer; this SNP is close to the POU5F1P1 transcription factor (pseudo-) gene and MYC, and is involved in predisposition to CRC through adenoma formation
- rs4939827, rs12953717 and rs4464148 (18q21.1), within intron 3 of SMAD7, a negative regulator of the TGF-beta signaling pathway
- rs4779584 and rs10318 (15q13.3), intriguingly mapping to the same site as the high-penetrance Hereditary Mixed Polyposis Syndrome locus, and close to the GREM1/BMP antagonist
- rs10795668 (10p14), in a ‘gene desert’ of several hundred kb
- rs3802842 (11q23), near the transcription factor POU2AF1
- rs16892766 (6q23.3), close to the Eih3 transcription factor
- rs9929218 (16q22.1), close to the E-cadherin gene
- rs961253, rs355527 (20p12.3), about 500 kb proximal to BMP2
- rs444235 (14q22.2), close to BMP4
- rs10411210 (19q13.11), close to rhophilin2

The relative risks conferred by these SNPs are typically of the order of 1.2-1.3 per allele, and the effects appear to be approximately additive. Although the SNPs only account for a small proportion of the familial relative risk of colorectal cancer, they do predict differential risks on a population level that are promising for use in clinical practice.

In addition, we are beginning to see evidence of effects of specific pathways on cancer susceptibility. Several colorectal cancer SNPs act in the BMP signalling pathway, which may be involved in maintenance of the stem cell niche in the colorectal crypt.

Our work has comprised three main strands, genetic susceptibility to cancer; functional genetics of colorectal tumorigenesis, and mitochondrial tumour susceptibility genes.
Functional genetics of colorectal tumorigenesis

One of the aims of the MPGL has been to characterise the genetic pathways of colorectal tumorigenesis, with emphasis on the co-selection of advantageous mutations and on the role of genomic instability. The APC gene has always been the most important focus of this work, because it is mutated somatically in over 70% of CRCs and there exists the disease familial adenomatous polyposis (FAP) in which patients carry germline APC mutations and develop multiple tumours. APC is frequently cited as a classical tumour suppressor gene (TSG), requiring two inactivating mutations to initiate colorectal tumorigenesis. However, several observations, including our own, have suggested that there is more to APC mutation than apparent at first sight.

In summary

- **germline APC mutations before exon 4, in exon 9 and distal to codon 1580 are associated with mild (attenuated) familial adenomatous polyposis (FAP)** (Spirio et al., 1993)
- **somatic APC mutations cluster in the region between codons 1280 and 1500** (Miyoshi et al., 1992)
- **LOH is uncommon at APC** (in both FAP and sporadic tumours) compared with other TSGs (Lamlum et al., 1999)
- **APC mutations in a small region close to codon 1309 are strongly associated with a 'second hit' by LOH**, whereas LOH is rare with other mutations and truncating 'second hits' are common (Lamlum et al., 1999; Rowan et al. 2000)
- **codon 1309 germline mutations usually cause very severe FAP** (Nugent et al., 1994)
- **LOH at APC occurs by mitotic recombination rather than by deletion** (Sieber et al., 2002)
- **APC mutation has a postulated role in CIN** (Fodde et al., 2001; Kaplan et al. 2001; Dikovskaya et al., 2007), but *FAP adenomas are (near-)diploid* (Sieber et al., 2002)
- **tumours of patients with attenuated FAP often harbour three hits at APC** (Spirio et al., 1998; Su et al., 2000; Sieber et al., 2006)
- **three hits affect APC in some sporadic CRCs** (unpubl. data)

To cut a long story short, we provided a general explanation for all these findings by showing that the two APC mutations in colorectal tumours are co-selected. The position of the 'first hit' determines the position and/or type of the 'second hit'. We postulated that the 'first hit-second hit' association occurred so as to produce an optimal level of beta-catenin protein and hence, optimally activated Wnt signalling. Sometimes, achieving this optimum required 'three hits' at APC. These observations were summarised as the 'just right' model of Wnt signalling in colorectal tumorigenesis.

Owing to intrinsic difficulties in studying sub-optimal combinations of APC mutations in human tumours, we have therefore proceeded to construct a small number of new models of intestinal tumorigenesis, to be analysed alongside data from human cancers and cell lines. We now have early data from our first model, Apc1322T (1322T), that carries an APC mutation close to the 1309 site and leaves one 20AAR in the stable, truncated protein. After repeated back-crossing to the C57BL/6j background, we compared the 1322T animals with the widely-used ApcMin (Min) mouse in which the mutant Apc protein has zero 20AARs. In both mice, intestinal adenomas showed copy-neutral loss of heterozygosity, making them homozygous for the mutant Apc allele. 1322T animals had markedly more severe polyposis, with earlier-onset, larger, more numerous, and more severely dysplastic adenomas. 1322T tumours also had more marked Paneth cell differentiation and higher frequencies of crypt fission. Somewhat surprisingly, however, nuclear beta-catenin expression was lower in 1322T tumours (left) than the Min tumours (right).

These results suggest that the Apc1322T mutation produces levels of beta-catenin (and hence, perhaps, of Wnt signalling) that are sub-maximal, but that promote early tumour growth more effectively than the ApcMin mutation. The 'just right' model of selection for specific combinations of APC mutations can be explained in this way.

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The autophagic response is regulated by the number of autophagosomes formed, and their rate of consumption by lysosomal degradation. The laboratory has been investigating both processes to understand the molecular mechanisms underlying the regulation of autophagy. We aim to achieve an understanding of these mechanisms allowing us to extend our findings to address the role of autophagy in human disease, in particular cancer.

**Autophagosome formation**

Amino acid starvation inactivates TOR (target of Rapamycin), a key growth promoting kinase and a negative regulator of autophagy. In yeast, the Atg1 kinase is downstream of TOR, and is proposed to control early events in autophagy. Ed Chan identified the Unc-51-like kinase 1 (ULK1) in a siRNA kinome screen as the mammalian homologue of Atg1 (Chan et al., JBC 282; 25464-25474), and showed that depletion of ULK1, but not the closely related ULK2, inhibited starvation-induced autophagy. To further understand the role of ULK1 in autophagy, Ed, in collaboration with Andrea Longatti and Nicole McKnight, uncovered an intra-molecular regulation of the ULK1/2 kinases through autophosphorylation, and modulated by a novel ULK1/2 substrate, KIAA0652, the putative orthologue of yeast Atg13. Mammalian Atg13 interacts with, and is a substrate of ULK1/2, and has a starvation-sensitive membrane association. Future work on ULK1/2 and mAtg13 aims to understand the regulation of this complex, and the amino acid signalling pathways controlling the expansion of the isolation membranes (IM) and nascent AVIs. Furthermore, Nicole McKnight, with the LRI High Throughput Screening Unit headed by Mike Howell, is conducting a siRNA genome screen for novel regulators of autophagy.

ULK1 is required for mammalian Atg9 trafficking to the autophagosome (Young et al, JCS 119:3888, 2006), and mAtg9 is the only transmembrane protein so far thought to be on IMs. mAtg9 cycles between the TGN and endosomes.
under normal conditions, and after starvation mAtg9 redistributes to autophagosomes. To understand how mAtg9 trafficking is controlled, Andrea Orsi investigates the cytoplasmic domains of mAtg9 to uncover trafficking signals and effectors, and together with Jemma Webber is exploring further the role of ULK1 in mAtg9 trafficking.

Jemma has recently identified a new mAtg9 partner, p38IP (p38α MAPK interacting protein), which is required for starvation-induced mAtg9 trafficking and autophagosome formation. Jemma’s hypothesis is that p38MAPK, a negative regulator of basal and starvation induced autophagy, and acts through mAtg9 and p38IP and provides a mechanistic link between the MAPK signalling pathway and the control of autophagy through mAtg9 trafficking via p38IP.

Formation of the IM requires the Class III Phosphatidylinositol-3-kinase (PtdIns-3-K). The production of PtdIns-3-phosphate is essential for the formation and expansion of the autophagosome. Beclin1 is an essential subunit of the PtdIns-3-K complex, and is required for autophagy. It is not known how PtdIns-3-P production is localised to the IM, and how the Class III kinase is regulated. Harold Jefferies is studying the composition and subcellular localisation of Beclin1-containing complexes to understand more about this issue. Furthermore, a family of PtdIns-3-P and PtdIns-3,5-P2 binding proteins, the WIPI family, are required for autophagosome formation although what their function is remains unknown. Hannah Polson (in a collaboration with Dr. M. Claque, University of Liverpool) has been studying this family to determine which WIPI proteins are recruited to the IM, and how. Additional information will be gained by Hannah through the identification of novel WIPI effectors.

**Autophagosome maturation and fusion**

In the final stages of autophagy, autophagosome fuses with endosomes and lysosomes and become degradative (AVds). How this fusion occurs is not known, although it is likely based on membrane fusion paradigms developed from the well-studied endosome-late endosome fusion. The laboratory has a long-standing interest in membrane fusion events in the secretory pathway, and Grant Otto has continued this interest with his work on the SNARE protein Syntaxin 6. Grant has characterised a novel Syntaxin 6-binding protein, KIAA0701, which he showed binds and regulates Syntaxin 6 function in endocytic recycling pathway. Grant’s work has potentially uncovered a novel regulation of SNAREs and membrane traffic.

Finally, we aim to understand more about autophagosome-endosome fusion. Minoo Razi has shown that early endosomes fuse with autophagosomes by preventing early endosome function by siRNA depletion of the coat protein complex COPI. Loss of COPI causes an accumulation of non-degradative autophagosomes, and an inhibition of autophagy. However, it is not clear if fusion with early endosomes is a pre-requisite for fusion of autophagosomes with late endosomes. Thus, to investigate the molecular requirements for autophagosome fusion with endosomes, Joëlle Morvan and Harold Jefferies have exploited an in vitro fusion assay that reconstructs AVi-E/LY fusion. They found this fusion event has several unique properties, the most significant being a lack of dependence on ATP, and suggest that the fusion between the autophagosome and the endosome is an unusual type of fusion. It remains to be understood if the SNARE fusion machinery is catalysing this fusion.

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**Figure 1.** The autophagy pathway in mammalian cells. Autophagy is induced on isolation membranes (IMs) that serve both as signaling platforms and membrane source/acceptor compartments. The source of the IM is unknown. During expansion, the IM grows sequestering cytosolic components. After closure of the expanded IM, the immature autophagosome (AVi) fuses with the endosomal compartments (E) and lysosomes (LY), becoming a degradative autophagosome (AVd). Degradation of the sequestered cytosolic components occurs in the AVd as it matures to an autolysosome. In the final stage, the AVd is shown in cross-section to aid visualisation of the membrane fusion event(s).
SRF interacts with members of two families of regulatory cofactors, which contact both its DNA-binding domain and the neighbouring DNA. The TCFs (Ternary Complex Factors: Elk-1, SAP-1, Net) are Ets domain proteins that link SRF activity to MAPK signalling pathways, while the MRTF (Myocardin-related Transcription Factors: MAL and Mkl2) link its activity to the dynamics of the actin cytoskeleton.

Changes in cell identity, proliferation and behaviours such as motility and adhesion are induced by extracellular signals which in many cases act by changing patterns of gene transcription. We study a network of transcription factors involved in all these processes, which controls the activity of the MADS-box transcription factor SRF (Serum Response Factor). Work this year has given new insights into molecular mechanism of actin-MAL interaction and functional redundancy amongst TCF family members, and has demonstrated that MRTF activity is required for numerous cellular behaviours that depend on actin cytoskeleton remodelling.

SRF network indirectly, by altering G-actin levels, which in turn appears to control the formation of actin-MRTF complexes. Central to this regulation is the highly conserved RPEL domain at the MRTF N-termini which contains three so-called RPEL motifs. With Stephane Mouilleron and Neil McDonald (LRI Structural Biology Group), Sebastian Guettler previously solved the structures of cocrytals between actin and either RPEL peptides or RPEL domain subfragments. The RPEL motif-actin interaction shares features with other actin-protein complexes and exhibits a striking similarity to the interactions with actin made by two discrete regions of vitamin D binding protein. Carola Langer used site-directed mutagenesis to verify that the interactions seen in the structure are functionally relevant. With Maria Wiezlak, Carola is working to examine the relevance to MAL regulation of larger actin-RPEL complexes, whose structures are currently under investigation.

Tamara Gruener has developed high throughput RNAi screens for the genetic identification of MRTF regulatory factors in drosophila tissue culture cells, in which the MRTF-SRF pathway is conserved. Working with Mike Howell in the LRI high-throughput screening facility, Tamara has identified a number of candidate regulatory factors in this screen, many of which are conserved through vertebrate evolution. The majority of these proteins appear to affect reporter activity but not MRTF localisation, but several appear to act at the level of MAL nucleocytoplasmic shuttling. Tamara is currently investigating the mechanism by which these proteins affect MRTF activity. Rafal Pawlowski has also continued studies on the role of phosphorylation in MAL regulation using phospho-specific antibodies and mass spectrometry. His data suggest that phosphorylation plays both positive and negative roles in transcriptional activation by MAL but does not affect its nucleocytoplasmic shuttling.
SRF-cofactor interactions

Classical studies in tissue culture cells suggested that immediate-early gene activation plays an essential role in cell cycle re-entry function, and immune cells in our TCF knockout animals provide an interesting system in which to study this. Patrick Costello and Diane Maurice find that while IE gene expression appears to sensitise some cell types to cell cycle re-entry in response to TCR ligation, in others it appears to inhibit it. Diane is currently investigating the molecular basis of these phenomena.

Cyril Esnault has worked with Rob Nicolas to develop chromatin immunoprecipitation approaches for the study of SRF-cofactor interactions in fibroblasts and immune cells, aiming to investigate the factors which determine specificity of cofactor recruitment for different SRF target genes. These methods will be used on a genome-wide scale to investigate cofactor activity during growth factor stimulation. Rob Nicolas’ array analysis of gene transcription following TCR activation in DP thymocytes demonstrated that the classical SRF-controlled IE genes are differentially sensitive to TCF deletion; with Cyril Esnault he has shown that this apparently reflects differential TCF recruitment to specific SRF target genes. Differential TCF recruitment may underlie Patrick and Rob’s finding that ectopic expression of Elk-1, but not Net, can rescue the thymocyte selection defect in animals lacking SAP-1. Anastasia Mylona has used SRF mutants to demonstrate that cofactor interactions are essential for T cell development, and is continuing this work by examining cofactor interactions using biochemical approaches.

The SRF network in cytoskeletal dynamics and metastasis

Rho GTPases control the dynamics of the cytoskeleton through multiple effector proteins that regulate the assembly, activity and turnover of actin-based structures, thereby controlling cell adhesion, morphology and motility. Numerous studies of both human cancers and mouse cancer models have implicated Rho signalling, and RhoC in particular, in cancer cell invasion and metastatic tumour spread, and this has been generally thought to reflect direct effects on the cytoskeleton. Although the control of MRTF activity by Rho potentially provides a mechanism by which cytoskeletal gene expression can be coordinated with cytoskeletal regulation, the notion that transcriptional regulation via the Rho-actin-MRTF-SRF nuclear signalling pathway is required in addition to Rho-controlled cytoplasmic events for effective execution of cytoskeletal remodelling has remained untested.

To address these issues directly and to elucidate its significance for tumour metastasis, Souhila Medjkane and Cristina Perez studied the role of MRTF-SRF signalling in two highly metastatic tumour cell lines, human MDA-MB-231 breast carcinoma and mouse B16F2 melanoma. Depletion of MRTFs or SRF using shRNA-mediated knockdown reduces cell adhesion, spreading, invasion and motility in culture, without affecting proliferation or inducing apoptosis. In studies carried out with Cedric Gaggioli and Erik Sahai, Souhila found that MRTF-depleted tumour cells were strikingly impaired in organotypic invasion models, and exhibited reduced cell motility in tumour xenografts, suggesting a defect in metastatic potential. Consistent with this, MRTF- and SRF-depleted tumour cells are unable to colonise the lung from the bloodstream in an experimental metastasis model, even though they initially arrive at the organ in similar numbers to non-depleted cells (Figure 1). Actin-based cell behaviour and experimental metastasis thus requires Rho-dependent nuclear signalling through the MRTF-SRF network. Microarray analysis identified Myh9 and Myl9 as among a small number of shared MRTF-SRF targets required for these processes. Jonathan Tobin and Victoria Lawson will take this work forward to investigate at which steps MRTF-SRF signalling is involved in the metastatic process in vivo.

Figure 1. MRTF-SRF signalling is required for experimental metastasis. Left, B16F2 mouse melanoma colonies established in the mouse lung by control and MRTF-depleted cells are visible as dark spots. Right, human MDA-MB-231 breast carcinoma cells depleted of SRF are greatly impaired in lung colonisation compared to control cells, as assessed following H+E staining of lung sections.

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Establishment of sister chromatid cohesion during DNA replication

During S-phase of the eukaryotic cell cycle, two identical copies of each chromosome are synthesised by DNA replication. These two copies, the sister chromatids, need to be faithfully distributed between daughter cells during mitosis. To make it possible for the mitotic machinery to recognise pairs of replication products for segregation, these are kept linked to each other by sister chromatid cohesion from their synthesis onwards until they split in anaphase. Sister chromatid cohesion is mediated by the chromosomal cohesin complex, a large ring-shaped multisubunit protein complex that is thought to bind to DNA by topological embrace. Cohesin is loaded onto chromosomes well before S-phase, but it is during S-phase that it establishes physical cohesion between the two newly replicated sister chromatids. One of the interests in our laboratory is to understand the reactions by which cohesin recognises and holds together sister chromatids during DNA replication.

We have in the past analysed what happens to cohesin as the replication fork travels along the DNA during S-phase. Like most other chromosomal proteins, cohesin may have to transiently dissociate from chromosomes as the fork approaches and DNA is replicated. In this case, cohesin needs to be loaded again onto DNA in a cohesive fashion in the wake of the replication fork. However, we found that the protein factors and reactions that load cohesin onto DNA before S-phase are no longer required during DNA replication. This suggests that reactions different from the initial loading reaction take place during S-phase, or that alternatively the replisome might be able to slide through the large cohesin rings to establish sister chromatid cohesion without displacing them from DNA. To distinguish between these two possibilities, and to gain insight into the process of cohesion establishment, we are drawing our attention to a group of proteins known as ‘cohesion establishment factors’.

In addition to the cohesin complex itself, a number of these additional cohesion establishment factors are required to ensure that cohesin links sister chromatids after DNA replication. Among these factors, the Eco1 acetyl transferase is of particular interest. Besides the subunits of the cohesin complex, Eco1 is the only other protein whose function is essential and therefore indispensable for the establishment of sister chromatid cohesion. We found Eco1 to be part of the replication fork machinery, but how it promotes sister
chromatid cohesion and what its acetylation target might be remained unknown.

Eco1-dependent cohesin acetylation during establishment of sister chromatid cohesion

To gain insight into Eco1 function, we isolated spontaneous suppressor mutations in budding yeast that allow cell growth after inactivation of the temperature sensitive eco1-1 allele, conditions that would normally lead to chromosome missegregation and cell death. Genetic analysis of the suppressors indicated that mutations in three different genes were able to restore viability to eco1-1 cells at the restrictive temperature. To identify the suppressor mutations, genomic DNA of the suppressors was hybridised to oligonucleotide tiling microarrays covering the budding yeast genome. Point mutations in unknown genes, should become detectable as reduced hybridisation efficiency due to the mismatch between the mutant genomic DNA and the 25-mer oligonucleotide probes present on the microarray. This strategy allowed us to identify two of the three suppressor mutations (Figure 1a).

One of the suppressors was a lysine to asparagine mutation (K113N) in Smc3, a subunit of the cohesin complex (Figure 1a). Lysines are the targets of acetylation, and an asparagine sidechain shares features with acetylated lysine. In a collaboration with the Protein Analysis at Clare Hall, we discovered that Smc3 is indeed acetylated at both lysine 113 as well as the neighbouring lysine 112 residue. Acetylation occurs in an Eco1-dependent reaction during the establishment of sister chromatid cohesion (Figure 1b). Preventing Smc3 acetylation by replacing lysine 113 with arginine interferes with cohesion establishment. In contrast, the acetylation-mimicking K113N mutation allowed cell growth and establishment of sister chromatid cohesion even in cells lacking the ECO1 gene altogether. This suggests that the essential function of Eco1 during DNA replication is acetylation of the cohesin subunit Smc3.

The significance of Smc3 acetylation became clear from analysis of suppressor mutations in the second complementation group, which inactivated the budding yeast ortholog of the cohesin destabilising protein Wapl. Just like the Smc3K113N mutation, inactivation of Wapl also made Eco1 dispensable for establishment of sister chromatid cohesion. It therefore emerges that an essential aspect of sister chromatid cohesion establishment is cohesin stabilisation against Wapl by acetylation of Smc3 (Figure 1c). These findings explain the essential role of Eco1 in sister chromatid cohesion.

Outlook

In the absence of both Eco1 and Wpl, the fundamental mechanism for pairing sister chromatids during DNA replication remains intact. This leaves open the important question how cohesin recognises and holds sister chromatids together. Reactions that are innate to the DNA replication process, for example passage of the replication fork through the cohesin ring, may provide the underlying basis for sister chromatid cohesion. We are now interested to learn how additional cohesion establishment factors that are associated with the replisome, including Ctf4 and Ctf18 (Figure 1c), act to promote cohesion establishment.

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Figure 1. Acetylation of the cohesin subunit Smc3 during cohesion establishment. a) Identification of the Smc3 K113N mutation. Genomic DNA of the eco1-1 parental strain and suppressor a (SUPa) was hybridised to whole genome oligonucleotide tiling arrays. Reduced hybridisation efficiency, as a function of the sequence context, was used to calculate the prediction signal for a base mismatch. The mutation was then confirmed by DNA sequencing. b) Smc3 acetylation at the time of cohesion establishment. G1 cells were released to progress through a synchronous cell cycle. FACS analysis of DNA content is shown, and the percentage of cells in anaphase (binucleates) is indicated. Smc3 was immunopurified from cell extracts at the indicated times via its Pk affinity epitope tag and analysed by Western blotting with α-Pk and α-acetyl lysine antibodies. c) Model for Eco1 function during establishment of sister chromatid cohesion. Wapl destabilises cohesin on chromosomes. To reach stable sister chromatid cohesion, Eco1 acetylates Smc3 as the replication fork passes, making cohesin resistant against Wapl.

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Regulation of ubiquitination

Given the complexity of the system it is unsurprising that there are many proteins involved in the regulation of ubiquitin modification. Ubiquitination is achieved through a hierarchical cascade of enzymes: E1, which activates Ub in an ATP-dependent manner; E2, which forms a thioester linkage with Ub; E3 which mediates the transfer of ubiquitin from E2 to the target, and therefore provides the substrate specificity. Many E3 enzymes are involved in disease states, particularly cancer, as their pivotal role in selection of substrate and mediation of the ubiquitination event is essential for the correct function of the pathways the substrate is involved in. The Protein Structure and Function lab investigates mechanisms of E3 action adopting a structural and biochemical approach.

Specificity and selectivity in the ubiquitin cascade

There are many mechanistic questions that surround the E3-substrate interaction and how specificity for both target protein and type of modification is achieved. We aim to answer these questions exploiting three model systems for the understanding of the molecular mechanisms of ubiquitination of a target molecule. One model in the lab is...
the Fanconi Anaemia pathway, which is a DNA repair pathway with monoubiquitination at the heart. Patients with Fanconi Anaemia are sensitive to DNA breakages, and have a high disposition to cancer. The central core of the pathway is a poorly characterised multi-subunit protein complex responsible for monoubiquitinating FANCD2 and FANCI at a specific site on each. There is no evidence of ubiquitin chain formation or other sites of ubiquitination, and therefore this pathway represents an ideal model for understanding substrate and ubiquitination specificity.

**The Fanconi-Anaemia pathway**

Fanconi Anaemia (FA) is a rare recessive disorder characterised by chromosomal instability and a pre-disposition to cancer. FA is divided into at least 13 complementation groups (FA-A, B, C, D1, D2, E, F, G, I, J, L, M and N). However, FA components are also implicated in other genome instability disorders such as Bloom’s syndrome. FA proteins are also closely related to the BRCA pathway which is found to be mutated in many cases of breast cancer – FANCD1 is BRCA2, FANCN is PALB2 (Partner and Localiser of BRCA2), and FANCJ is identical to BACH1/BRIP1, a DNA helicase that interacts directly with BRCA1. A, B, C, E, F, G, L, M and 2 uncharacterised components FAAP100 and FAAP24 form the nuclear FA core complex (Figure 1.) This complex is required for monoubiquitination of FANCD2 on lysine 561 and lysine 523 of the newly-identified FANCI, so currently there are 2 substrates for its monoubiquitination activity, but FANCI and FANCD2 share 40% sequence similarity in the region surrounding the ubiquitination site. The discovery of FANCI as a target for the complex opens up a whole new set of questions regarding the machinery, and it is still unclear what regulators of the activity of the complex might be, although Ube2T was recently predicted to be the E2 for the pathway.

Of the 10 components of the core complex, only M and L have identifiable domains. FANCM has DNA helicase-like and endonuclease-like domains, suggesting it may be involved in processing DNA, and FANCL has a RING finger domain and WD40 repeats suggesting it may be the catalytic ‘E3’ component of the complex.

We are aiming to structurally characterise the whole core complex, in order to understand how the complex recognises and ubiquitinates FANCD2 (and FANCI), and how this event is compromised, on a molecular level, in the disease state.
Signalling dynamics and regulation of actin based motility

Understanding how signalling networks regulate cell motility and adhesion will require a complete molecular understanding of the protein interactions occurring within each signalling network. It also requires detailed knowledge of the cellular localisation and dynamics of individual components and/or protein complexes within each network. Unfortunately, many signalling networks are often not amenable to such analysis, as they are frequently transient and dispersed. In contrast, many of the signalling pathways that are hijacked and manipulated by pathogens undergoing actin-based motility, are highly localised and sustained.

During vaccinia virus infection the extra-cellular enveloped virus attached to the plasma membrane induces an outside-in signalling cascade that locally activates Src and Abl family kinases. This activation results in phosphorylation of tyrosine 112 and 132 of A36, an integral viral membrane protein that is localised beneath the extra-cellular virus. Phosphorylation of tyrosine 112 creates a binding site for the SH2 domain of the adapter Nck, which is recruited beneath the extra-cellular virus as part of a tripartite complex that contains WIP and N-WASP. Phosphorylation of tyrosine 132 generates a binding site for the SH2 domain of the adapter Grb2. The recruitment of Grb2 however, requires the prior phosphorylation of tyrosine 112, as its association also depends on the presence of the polyproline rich region of N-WASP. Ultimately the recruitment of N-WASP locally stimulates the actin-nucleating activity of the Arp2/3 complex, resulting in the formation of an actin tail beneath the virus, which acts to enhance viral spread.

The signalling network vaccinia hijacks to stimulate actin polymerisation is also involved in a number of cellular processes such as the formation and regulation of invadopodia during tumour cell-induced matrix degradation and invasion. The highly localised and robust nature of the vaccinia signalling cascade make the virus a power model system to understand the molecular details of how a signalling network induces and regulates actin-based motility.
virus was found to be dependent on actin polymerisation nucleated by the Arp2/3 complex. This suggests there is a feedback mechanism, in which active actin polymerisation regulates the exchange of the vaccinia-signalling complex. Consistent with this hypothesis, the stability of N-WASP beneath the virus depends not only on its interaction with Grb2 but also the growing barbed ends of actin filaments. Disruption of either of these interactions leads to an increase in the rate of N-WASP exchange, which in turn results in a faster rate of virus movement. Our observations are consistent with a model in which the stability of N-WASP association regulates the rate of Arp2/3 complex-dependent actin-based motility by antagonising actin filament capping. Our future studies will be aimed at understanding the relationship between the stability of N-WASP, barbed end capping and Arp2/3 complex-dependent actin filament nucleation.

**An E2-F12 complex is required for IEV morphogenesis during vaccinia infection**

Vaccinia actin tail formation only occurs after intra-cellular enveloped virus (IEV) particles have fused with the plasma membrane. In order to reach the plasma membrane from their peri-nuclear site of assembly, IEV particles recruit kinesin-1 and undergo microtubule dependent movements towards the cell periphery. Currently, only two viral proteins, A36 and F12, have been suggested to play a direct role in microtubule mediated movement of IEV. Loss of either A36 or F12 results in the accumulation of IEV at their peri-nuclear site of assembly. A36 appears to be responsible for recruiting the microtubule motor as it can interact directly with the kinesin-1 light chain. In contrast, the role of F12 in the movement of IEV towards the plasma membrane remains obscure.

Over the last year Mark Dodding, a postdoc in the laboratory, has been imaging live cells infected with a recombinant virus expressing GFP-F12 to obtain additional insights into role of F12 during IEV transport. Mark found that GFP-F12 is recruited to IEV moving on microtubules and is released from virus particles when they fuse with the plasma membrane and switch to actin-based motility. Unexpectedly, Mark found that although the majority of IEV remain close to their peri-nuclear site of assembly in the absence of F12, a small number of IEV are still capable of undergoing microtubule mediated transport. Using a recombinant virus expressing GST-F12 in conjunction with mass spectrometry analysis of glutathione resin pull downs, Mark was able to demonstrate that the viral protein E2 interacts directly with F12. In infected cells, GFP-E2 is observed on IEV moving on microtubules as well as in the Golgi region, but is not associated with actin filaments. In the absence of the gene encoding E2, IEV accumulate in the peri-nuclear region and F12 is not recruited to virus particles. Conversely, GFP-E2 is not observed on IEV in the absence of F12. Ultra-structural analysis of cells infected with viruses lacking the genes encoding E2 or F12 by Lucy Collinson in the Electron Microscopy Unit revealed that loss of either protein results in defects in membrane wrapping during IEV formation. Taken together, our observations suggest that the primary role of the E2-F12 complex is in the morphogenesis rather than the microtubule-based transport of IEV.
We have continued to investigate a number of themes, the common thread being the role of adult stem cells in neoplasia. How mutations are fixed and spread within the gastrointestinal tract, particularly in pre-malignant conditions, is poorly understood and has important implications for carcinogenesis.

We have used Barrett’s oesophagus and ulcerative colitis to analyse how a mutated clone with a selective advantage can clonally expand to fill an entire segment of mucosa at the expense of competing clones (selective sweep to fixation model), assessing clonality at a high resolution by micro-dissecting and genetically analysing individual crypts. In colitis-associated neoplasias we found that in most lesions an oncogenic mutation could be identified in all crypts across the lesion showing monoclonality. This founder mutation was a \( p53 \) lesion in the majority of neoplasms but four tumors had an initiating \( K-RAS \) mutation. Some nondysplastic crypts surrounding areas of dysplasia were found to contain clonal \( p53 \) mutations and in one case three clonal tumors arose from a patch of nondysplastic crypts containing a \( K-RAS \) mutation. Thus \( p53 \) mutation is initiating mutation in the majority of lesions, but \( K-RAS \) activation as an alternative gatekeeping mutation and we also showed local and segmental field cancerisation was present by showing pro-oncogenic mutations in nondysplastic crypts surrounding neoplasms. In contrast, in Barrett’s oesophagus we found marked clonal heterogeneity, with multiple independent clones present, showing that that Barrett’s heterogeneity arises from multiple independent clones, in contrast to the selective sweep to fixation model of clonal expansion previously described. We identified a \( p16 \) point mutation arising in the squamous epithelium of the oesophageal gland duct, which was also present in a contiguous metaplastic crypt, whereas neo-squamous islands arising from squamous ducts were wild-type with respect to surrounding Barrett’s dysplasia. It appears that the squamous gland ducts situated throughout the oesophagus are the source of a progenitor cell that may be susceptible to gene mutation resulting in conversion to Barrett’s metaplastic epithelium. Additionally, these data suggest that wild-type ducts may be the source of neo-squamous islands. Additionally, we have found a patch of intestinal metaplasia in the stomach which shows a clonal \( APC \) mutation, further establishing the clonal nature of the lesion and developed a computational framework to study the balance between the mutation rate and the balance between positive and detrimental selection during cancer growth.

We have built on our understanding of the early stages of development of polyps in human familial adenomatous polyposis and in \( Apc\)\((\text{min}^+/+)\) mice that model this genetic susceptibility to cancer: early lesions in humans and mice with abnormalities in the gene, and tested in \( Apc\)\((\text{min}^+/+)\) mice certain inhibitors of tyrosine kinases that are in clinical trials for human colorectal cancer. We have also have established a protocol in the lab for sequencing methylation patterns from laser-capture micro-dissected human epithelial tissue. In collaboration with the Tomlinson lab, we carried out an analysis of expression of 22 genes using in situ hybridisation showed that the situation in vivo was more complex than apparent from preliminary validation by quantitative RT/PCR. Several of the newly identified, differentially expressed genes represent potential diagnostic or therapeutic targets for intestinal tumours.
We have continued to use mtDNA mutation analysis to locate the stem cell niche and to tracing cell lineages several human tissues. In the liver we have shown that clonal proliferative units exist in the human liver, an origin from a periportal niche is most likely, and that the trajectory of the units is compatible with a migration of cells from the periportal regions to the hepatic veins. In the pancreas, exocrine tissue progenitors appeared to be located in interlobular ducts. In the skin, the origin of a basal cell carcinoma appeared to be from the outer root sheath of the hair follicle, establishing this as general method in which stem cell niches and stem cell progeny can be recognised, also affording the generation of cell fate maps, all in human tissues. This technique also allows analysis of the origin of human tumours from specific tissue sites.

Continuing our research into the BM-derivation of renal epithelium, we have shown that mesenchymal stem cells isolated, cloned, and cultured but retaining tri-lineage potential, do not engraft or fuse to assist renal regeneration after injury causing acute tubular necrosis; in contrast, co-administered MSC depleted whole BM does contribute to regeneration. Our recent results suggest that β3-integrin plays an important role in the engraftment of BMDC and angiogenesis and that systemically administered MSCs are recruited to and engraft at sides of inflammation in experimental colitis.

We have continued our attempts to over-express the human gastrokine-2 protein using a variety of techniques and intend to use the protein to look at interactions with TFF2.

With support from Dr Michael Ellis of Digital Scientific (Cambridge UK) we tested a tuneable selective emission filter for fluorescence microscopy and found this could be used to improve signal specificity when using complex chromophores with broad spectra that are commonly used for immunohistochemistry, but interfere with dyes often used for FISH probes.

Figure 1. Colonic epithelium in a female recipient mouse, 38 days after bone marrow transplant from a male donor mouse. Paraffin section was stained for endothelial cells with the antibody endomucin, developed with DAB (black), followed by in situ hybridisation for chromosomes, X (Cy3) and Y (FITC). Overlay photomicrograph using the DAB signals to align the endothelial cells. First photo: brightfield with DAB (black), nuclei (haematoxylin), Cy5 fluorescence (false-coloured the nuclei magenta), and aqua for background morphology. Second photo after XY FISH. White arrow: donor-derived male endothelial cells. Black arrow: possible donor-derived male epithelial cells. White double arrow: donor-derived male pericryptal myofibroblasts.

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DNA is highly susceptible to damage and must be repaired correctly to prevent loss of vital genetic information. Failure to correctly sense and/or repair DNA damage is the underlying cause of a number of hereditary cancer predisposition syndromes such as Fanconi anemia (FA) and Blooms. We are employing a number of complementary experimental systems, including *C. elegans*, mouse models and mammalian cell culture, to understand how DNA lesions are sensed and repaired during S-phase and in meiosis. We are particularly interested in understanding the function and regulation of the S-phase checkpoint, which senses replication stress, and Fanconi anemia (FA) and homologous recombination (HR) repair pathways.

**FANCM-FAAP24 facilitate ATR signaling**

The replication stress response pathway is a critical surveillance mechanism that coordinates the cellular response to replication stress. Central to this response is the ATR kinase, which is recruited to and activated by stalled replication forks. We have recently identified and partially characterised HCLK2 (also known as Tel2), which interacts with and regulates the stability of all PI3K-related kinases (PIKKs), including ATR. Through its association with ATR, HCLK2 is required for the replication stress checkpoint and activation of the FA and HR pathways. To better understand how HCLK2 functions, we have carried out proteomic analyses of purified HCLK2 complexes. In addition to previously reported interactions (e.g. ATR, DNA-PKcs) we also identified an association between HCLK2 and FANCM-FAAP24, the chromatin-targeting component of the FA core complex. Our studies revealed that in addition to defects in FA pathway activation, down-regulation of FANCM or FAAP24 also compromises ATR/HCLK2-mediated checkpoint signalling leading to increased endogenous DNA damage and a failure to efficiently invoke cell cycle checkpoints. Previous studies have shown that FANCM possesses DNA translocase and branch migration activities, which may function to remodel stalled replication forks. Intriguingly, we were able to show that the DNA translocase activity of FANCM, whilst being dispensable for FA pathway activation, is required for its role in ATR/HCLK2 signalling. Our data suggest that DNA damage recognition and remodelling activities of FANCM-FAAP24 co-operate with ATR/HCLK2 to promote efficient activation of DNA damage checkpoints (Collis et al, Mol. Cell 2008). More recently we have found that HCLK2 is subjected to various post-translational modifications. Investigations are underway to determine the functional relevance of these modifications and how they impact on the checkpoint and repair functions of HCLK2.

**PBZ: a novel Poly(ADP-ribose)-Binding Zinc finger motif in DNA repair/checkpoint proteins**

Poly(ADP-ribosyl)ation (PAR) is one of a large number of post-translational modifications of proteins that play an important role in mediating protein interactions and/or the recruitment of specific protein targets. PAR often involves the addition of chains of ADP-ribose units linked via glycosidic
ribose-ribose bonds, and is critical for a wide range of fundamental processes but is best known for its role in DNA repair. PAR synthesis is very rapidly induced at DNA damage sites where it is believed to promote recruitment of DNA repair factors (Figure 1). Targeting of proteins to these sites is dependent upon the efficient recognition of PAR by defined PAR-binding motifs or modules. We have recently identified a novel zinc finger motif, a Poly(ADP-ribose)-binding Zinc finger (PBZ), in a number of eukaryotic proteins involved in the DNA damage response and checkpoint regulation. We were able to demonstrate interaction of poly(ADP-ribose) with this motif in two representative human proteins, APLF (Aprataxin PNK-Like Factor) and CHFR (Checkpoint protein with FHA and RING domains), and show that the actions of CHFR in the antephase checkpoint are abrogated by mutations in PBZ or by inhibition of poly(ADP-ribose) synthesis. PBZ provides the first description of a zinc finger that binds poly(ADP-ribose), which is also required for post-translational poly(ADP-ribose) modification (Ahel et al., Nature 2008). Current studies are focused on characterisation of a novel PAR binding protein (PAR-BP) (Figure 1) that appears to play important roles in DNA repair and transcription.

RTEL1 is an anti-recombinase that impacts on genome stability and cancer

Unscheduled or excessive HR can lead to gross chromosomal rearrangements characteristic of cancer cells, but the mechanisms that restrain HR remain poorly understood. The Yeast Srs2 helicase suppresses aberrant recombination by disrupting a specific step in HR, however functional homologues are not obviously conserved in higher eukaryotes. We recently described a genetic screen in C. elegans to identify uncharacterised helicases that are synthetic lethal in combination with C. elegans BLM mutants, based on the srs2 sgs1 (BLM) synthetic lethality observed in yeast. This screen identified a novel helicase, RTEL-1 that is conserved from C. elegans to humans and exhibits many of the genetic and biochemical hallmarks of yeast Srs2 including hyper-recombination and exquisite sensitivity to various DNA damaging agents. Support for an anti-recombinogenic function for RTEL1 has come from biochemical studies. Purified human RTEL1 can actively disassemble D-loop recombination intermediates in an ATP-dependent manner. Previous work has shown that Rtel knockout mice die between days 10 and 11.5 due to dramatic genome instability and rapid telomere loss and Human RTEL1 is over-expressed in gastric tumours. Collectively, our data indicate that the phenotypes observed in C. elegans, mice and human cells are likely caused by a failure to correctly regulate HR. Promiscuous disassembly of recombination intermediates is also the likely underlying cause of genome instability in RTEL1 over-expressing cancers (Barber et al., Cell 2008). Current work is exploiting proteomic approaches, C. elegans synthetic lethal screens and conditional Rtel knockout/over-expressing mice to investigate the role of RTEL1 during meiotic recombination, DNA replication, telomere maintenance and tumourigenesis.

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Figure 1. Poly(ADP-ribosyl)ation (PAR) is a post-translational modification that is rapidly induced at sites of DNA damage. Poly-ADP-ribose polymerase (PARP) utilise NAD+ and ATP to catalyze the formation of PAR chains on protein acceptors that act to recruit PAR-binding proteins (PAR-BP) to damage sites. PAR chains are degraded by PARG.
Activating the DNA damage response

A regulatory network of proteins has been identified that participate in DNA damage checkpoint pathways. Central to this network are the ATM, ATR and the Mre11/Rad50/Nbs1 (MRN) complex. We are taking advantage of the vertebrate Xenopus laevis egg extract to study the biochemistry of the ATM, ATR and the MRN complex-dependent DNA damage response. In the recent years we have established several assays based on cell-free systems derived from Xenopus eggs to elucidate the biochemical bases of cell cycle checkpoints. We are using the Xenopus cell-free system in which we have already characterised ATM, ATR and the MRN complex to dissect the signal transduction pathway that senses DNA damage. Using this system we can characterise aspects of the checkpoint not easily accessible in other systems such as the study of the DNA lesions and the very early events responsible for the activation of the response. Among the cofactors required for ATM activation the MRN complex, which possesses nuclease activity, plays a major role. Addition of DNA DSBs to Xenopus egg extract leads to MRN-dependent ATM activation. Nuclease-dependent resection of DSBs appears to be important for the activation of the DNA damage response. We have obtained evidence suggesting that limited resection of DSBs is required to sustain ATM activity. In particular, we have shown that DSB processing depends on the endonuclease activity of the MRN complex, which resects ssDNA that progressively becomes available through the action of an associated helicase moving in the direction of the resection. As a result of this processing small single-stranded DNA oligos (ssDNA oligos) are generated. These ssDNA oligos participate in the activation of ATM in response to DSBs (Figure 1). ssDNA oligos produced at DSBs probably interact with one or more subunits of the MRN complex that have DNA-binding domains and perhaps other ssDNA binding proteins such as SSB1 to promote continuous stimulation of ATM molecules. Active MRN complexes can then facilitate the activation of inactive ATM molecules that have not yet engaged with DSBs amplifying the response. The creation of ssDNA oligos during the resection of DNA undergoing repair, either from 5’ to 3’ processing of DSBs or possibly from enlarging gaps in other forms of DNA repair is a unique signal of DNA damage. Whereas mononucleotides are produced by normal DNA metabolism, these ssDNA oligos form only during DNA damage processing and represent ideal alarm molecules that could work as second messenger signaling the presence of severe DNA damage. We are now testing the possibility to exploit this second messenger of DNA damage response to induce permanent cell cycle arrest such as senescence without damaging the genome. This strategy could be helpful in arresting the hyper-proliferation of cancer cells.
Identification of novel targets of the ATM/ATR dependent DNA damage response

The response to DNA damage is complex in vertebrates and some genes such as BRCA1 and p53 are present only in high eukaryotes. We are screening an expression library made of *Xenopus laevis* cDNAs to identify vertebrate targets of ATM and ATR. cDNAs can be in vitro transcribed and translated. Translated proteins are then mixed with extracts supplemented with damaged DNA, which is capable of activating ATM and/or ATR. Following incubation in extracts the protein mixture is separated on SDS-PAGE electrophoresis. Labeled proteins that undergo DNA damage dependent post-translational modifications are then isolated and characterised. This innovative strategy is allowing the rapid identification and cloning of proteins that are modified in the presence of active ATM and ATR. This screening has recently led to the isolation of the a centrosome protein that we named XCRA1 (Centrosome protein Regulated by ATM/ATR) as an ATM and ATR target involved in mitosis progression control following DNA damage. We have tested the effects of DNA damage occurring when cells are already in mitosis, a condition defined as mitotic catastrophe.

Xenopus egg extract is a powerful model system to study mitotic events such as spindle assembly. Addition of sperm nuclei to mitotic egg extract induces formation of half and full spindles. We found that ATM and ATR activation abolished spindle assembly inducing structures resembling aggregates formed by microtubules associated with chromosomal DNA. Inhibition of spindle assembly was dependent upon ATM and ATR. We then showed that XCRA1 is required for spindle assembly and that ATM/ATR dependent phosphorylation of XCRA1 inhibits spindle assembly. The checkpoint that we described operates in mitosis and might become important when other mechanisms preventing mitosis entry have failed. We now intend to characterise the molecular mechanism underlying the role of XCRA1 in spindle assembly. In particular, we would like to identify the proteins interacting with XCRA1 to see if the interaction is affected by the phosphorylation. Interestingly, human CRA1 is downregulated in several tumours suggesting that this protein is a novel tumor suppressor.

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DNA damage checkpoints and replication forks
Eukaryotic cells employ specialised surveillance mechanisms called ‘checkpoints’ to preserve both viability and genome integrity when confronted with endogenous or exogenous agents that interfere with DNA replication. The loss of checkpoint pathways can lead to genomic instability and thereby promote carcinogenesis. We previously found a role for DNA damage checkpoints in maintaining the stability of replication forks that encounter DNA damage. We provided evidence that this role explains why checkpoint mutants are hypersensitive to many DNA damaging agents. The DNA damage checkpoint involves a protein kinase cascade and, in Saccharomyces cerevisiae, the central checkpoint protein kinases are the ATR homolog Mec1 and its downstream effectors, Chk1 and the Chk2 homolog Rad53. In response to S-phase perturbations, Mec1 is recruited to stalled DNA replication forks where it is required to phosphorylate and activate Rad53. How these kinases then act to stabilise DNA replication forks has been somewhat mysterious, but is likely to have important implications for understanding how genomic instability is generated during oncogenesis and how chemotherapies that interfere with DNA replication might be improved. During this past year we showed that the sensitivity of rad53 mutants to a variety of DNA damaging agents can be almost completely suppressed by deletion of the EXO1 gene. Deletion of EXO1 also suppressed DNA replication fork instability in rad53 mutants. Deletion of EXO1, however, was completely ineffective in suppressing both the sensitivity and replication fork breakdown in mec1 mutants indicating that Mec1 has a genetically separable role in replication fork stabilisation from Rad53. This analysis also showed that the second downstream effector kinase, Chk1, which had not previously been implicated in budding yeast S phase responses to DNA damage, can stabilise replication forks in the absence of Rad53 (Figure 1). Our results reveal previously unappreciated complexity in the downstream targets of the checkpoint kinases and provide a framework for elucidating the mechanisms of DNA replication fork stabilisation by these kinases. Down-regulation of DNA damage checkpoints is believed to occur in many cancers and may help to explain why many cancers are responsive to chemotherapies that act by damaging DNA. Our results suggest that the loss of function of nucleases like Exo1 may represent a novel mechanism by which cancer cells become resistant to these drugs.

Factors influencing DNA double strand break response
Double strand breaks (DSBs) in DNA are amongst the most dangerous of chromosomal lesions, and can lead to cell death and genomic rearrangements. Two major pathways,
non-homologous end-joining (NHEJ) and homologous recombination (HR), compete for the repair of DSBs. During the first step of HR, breaks undergo nucleolytic degradation of their 5’-ending strands, a process known as resection. This generates 3’-ended single-stranded tails, which are required for the downstream events in HR. Resection also generates DNA end structures that are not used efficiently in NHEJ thus contributing to the switch between repair pathways. The choice between NHEJ and HR is also regulated by cyclin-dependent kinases (CDKs), and is therefore influenced by cell cycle stage. Cells are proficient for NHEJ in G1 when CDK activity is low, but not in G2/M, when CDK activity is high and HR is predominant. The molecular mechanism underlying these CDK-dependent effects is important but still obscure. In this past year, we described a novel quantitative assay to analyse DSB processing in the budding yeast, *Saccharomyces cerevisiae*. Consistent with previous work, we found evidence for extensive resection (>10 kb) from a DSB induced by the site specific HO endonuclease. However, our quantitative assay showed that only a small fraction of breaks are resected to this extent. This, together with previous genetic analysis suggests that resection may not be the only pathway for generating the ssDNA required for HR. We suggest that unwinding by an unidentified DNA helicase may be important for this. Our analysis also provided the first evidence for significant instability of the 3’ ssDNA tails. Thus, resection is not limited to the 5’ strand: the 3’ strand is also degraded, although this resection lags behind the 5’ strand resection. Resection of the 3’ strand might aid recombination or single strand annealing by ensuring there is a 3’ end close to the region of invasion/annealing. We also showed that both DSB resection and checkpoint activation are dose-dependent, especially during the G1 phase of the cell cycle. During G1, processing near the break is inhibited by competition with NHEJ but extensive resection is regulated by an NHEJ-independent mechanism. DSB processing and checkpoint activation are more efficient in G2/M than in G1 phase; however, we found that both processes are by far most efficient during S phase. We showed that this enhanced response requires that DNA replication forks encounter the break.

Our work in 2008 has helped identify important connections between DNA replication and DNA damage responses. This work was done primarily in budding yeast, and it will be important to examine these links in human cells to determine whether they have roles in cancer biology.

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![Figure 1](image.png)  
Figure 1. Model describing the role of DNA damage checkpoint kinases in regulating DNA replication fork stability after encountering a lesion (red X) in budding yeast. See text for details.
During the past year, we have made some good progress towards understanding how the process of cell division is initiated and terminated. In the Annual Report for last year, we said that we had discovered a phosphatase activity that reversed the action of mitotic cyclin-dependent protein kinases, whose activity was high in interphase (when the cells grow) and low in mitosis (when the cells divide), but we did not know its identity. Now we do, thanks to the investigations of Satoru Mochida, who simply asked which protein phosphatase activity was responsible. He raised antibodies against the catalytic subunits of the protein phosphatases present in frog egg extracts, and asked which of them removed ‘phosphatase X’ activity. It turned out that the antibodies against protein phosphatase 2A (PP2A) did not work very well for this purpose, but Gernot Walter and his colleague Ralf Ruediger kindly provided a monoclonal antibody against the A (scaffolding) subunit of PP2A, which works well in immunodepletion. This showed that phosphatase X was some form of PP2A. PP2A also contains a B subunit, of which several different varieties are known, so Satoru raised antibodies against all those he could find in the frog egg, and together with a summer student, Satoshi Ikeo, found that depletion of B-delta removed the majority of phosphatase X. What happens when this form of PP2A is removed from an extract that is running through the cell cycle? The answer, as can be seen in Figure 1, is quite dramatic. First, entry into mitosis is greatly accelerated, and subsequently, the extracts are stuck permanently in mitosis.

To confirm that this really was due to the loss of PP2A-Bδ, Satoru had to make an active preparation of the enzyme by expressing recombinant subunits in bacteria and insect cells, and then show that adding the synthetic enzyme back to the depleted extracts restored their normal cell cycle entry and exit. It took some time to achieve this end, and here we were greatly helped by Yigong Shi and his colleagues in Princeton who recently solved the structure of this form of PP2A. They generously provided clones and protocols for expression and assembly of trimeric PP2A complexes. Sure enough, when recombinant PP2A-Bδ is added back to the depleted extracts, they behaved exactly as normal undepleted extracts (Figure 1). Adding back too much PP2A-Bδ either slowed down entry into mitosis, or prevented it entirely (data not shown). These results clearly demonstrate that the level of PP2A-Bδ activity regulates the timing of entry into mitosis, and that this particular phosphatase is also necessary for exit from mitosis. These are two somewhat different things, and at the moment we do not know exactly how to interpret the results. We can also see from more detailed analysis of various parameters in the extracts that some subtle effects occur in extracts with too much or too little PP2A-Bδ. For example, entry into mitosis is marked by loss of tyrosine-15 phosphorylation of CDK1, in turn reflecting the balance of inhibitory Wee1/Myt1 kinase and its antagonist, the Cdc25 phosphatase. Probably, dephosphorylation of this pair of enzymes keeps CDK1 inhibited, whereas their phosphorylation tends to activate CDK1, regardless of any
effects on the substrates of CDK1. Thus, depletion of PP2A-Bδ probably helps to activate CDK1, and then makes it quicker and easier for CDK1 to work (because of the absence of the antagonising phosphatase). It is hard to disentangle these effects. Similarly, we note that the activation of the APC/C and subsequent degradation of cyclin B occur on time in relation to the activation of CDK1 in the PP2A-Bδ depleted extracts, but the inactivation of APC/C to allow the re-accumulation of cyclins to drive the next cell cycle is not quite right. At higher levels of added PP2A-Bδ, both activation and inactivation of the APC/C are seriously affected. This is going to be very complicated to work out, and our main task now is to discover the molecular basis for the mitotic inactivation (and post-mitotic reactivation) of PP2A-Bδ. We now have the tools to pursue this, and plenty of ideas about what might be happening, but no data yet.

Meanwhile, there has been progress on other projects. For example, Tohru Takaki, who was a postdoc in this laboratory before he joined Mark Petronczki’s lab, made a beautifully pure preparation of cyclin D3/CDK4 that enabled our friends in Oxford, Martin Noble and Jane Endicott and their colleagues, to obtain crystals and a structure for the complex. Although the preparation was largely phosphorylated and active as a protein kinase, it was the unphosphorylated form of the enzyme that crystallised. After dephosphorylation with λ-phosphatase, all residual activity is lost, but can be restored by CDK7/cyclin H. This is the first available structure for cyclin D, but we are slightly disappointed that the structure is of an inactive form of the enzyme.

Julian Gannon has been pursuing his studies of the activation of the APC/C, focussing on the role of phosphorylation of its activator, Cdc20. He has raised a number of phosphate-specific antibodies corresponding to sites in its N-terminus that are thought to play regulatory roles. Some of these sites (e.g. S50 and T79) show reversible phosphorylation when Xenopus egg extracts enter mitosis, and lose the phosphate upon return to interphase, but S114 is always phosphorylated. It is still extremely difficult to make active recombinant Cdc20 (except by translation of mRNA in vitro), which hampers our efforts to understand the regulation and role of this important activator of the APC/C.

Alessia Errico continues her work on Tipin and its role in DNA replication. She and members of Vincenzo Costanzo’s laboratory learned how to ‘comb’ DNA to visualise replication forks in single DNA molecules.

Publications listed on page 132
Skin cancer is the most frequently diagnosed malignancy. The UVB (wavelength 280-320nm) component of incident sunlight is an acknowledged contributor to skin carcinogenesis in the general population. The energy from UVB is directly absorbed by DNA bases and causes potentially mutagenic and cytotoxic photochemical DNA damage. Most (>95%) of the ultraviolet light that reaches the earth’s surface is the longer wavelength UVA (320-400nm), however. This is poorly absorbed by DNA and consequently is less directly harmful. Because of the abundance of UVA in incident sunlight, any therapeutic treatment that renders DNA vulnerable to damage by UVA might be expected to increase the risk of skin cancer. Some drugs introduce UVA chromophores into DNA. These permit the absorption of energy from these longer wavelengths. The thiopurines (6-thioguanine (6-TG), 6-mercaptopurine (6-MP), and azathioprine) which have been used for several decades as anticancer and anti-inflammatory agents, provide an example of this. Azathioprine is a widely-prescribed immunosuppressant in organ transplant patients. This patient group, which requires long-term treatment with immunosuppressant drugs to ensure the survival of the engrafted organ, suffers a hugely elevated incidence of non-melanoma skin cancer in which sunlight exposure is a major contributory factor.

Thiopurines cause 6-TG, a purine analog, to accumulate in patients’ DNA. We previously reported that DNA 6-TG absorbs UVA radiation and produces singlet oxygen (\(1O_2\)), a form of reactive oxygen that is highly damaging to both DNA and proteins. Using cultured cells containing DNA 6-TG, we demonstrated that DNA 6-TG is an important target for damage by \(1O_2\) and identified guanine sulfonate (GSO3) as a significant DNA photoproduct. We also showed that the PCNA DNA replication and repair factor is vulnerable to damage by \(1O_2\) generated by the interaction of UVA with DNA 6-TG. These DNA photoproducts are refractory to excision by DNA repair enzymes and have profound inhibitory effects on replication and transcription.

We have continued to investigate the photochemical reactions of DNA 6-TG. In particular, analysis by Xiaolin Ren and Feng Li, (in collaboration with Yao-Zhong Xu, Open University) of the stoichiometry of the UVA-mediated destruction of 6-TG has identified a second photoproduct, guanine sulfinate (GSO2), as an intermediate in the oxidation of 6-TG to GSO3. The stepwise oxidation of DNA 6-TG to GSO3 is prevented by some free radical scavengers such as ascorbate although other antioxidant compounds form addition products with intermediates of 6-TG oxidation.

The photochemical vulnerability of DNA 6-TG reflects two...
things: its ability to absorb energy from UVA to generate \(^{1}O_2\) and its relatively low oxidation potential. The latter property makes 6-TG extremely vulnerable to chemical oxidation and by reactive oxygen released by immune effector cells. 6-TG is incorporated freely into DNA of mitochondria. Mitochondria are potent sources of endogenous reactive oxygen species and Ilse has also shown that mitochondrial DNA of 6-TG-treated cells rapidly accumulates high levels of GSO3. This is associated with mitochondrial malfunction and cell death and defines a novel mechanism by which the thiopurines may be cytotoxic.

The 6-TG/UVA-mediated damage to proteins has been further investigated. Azadeh Kia together with David Frith (2-D Gel Electrophoresis Laboratory, LRI Lincoln’s Inn Fields), has examined changes in proteins induced by 6-TG/UVA treatment. In these experiments, altered proteins were identified by 2-D gel analysis of colour-derivatised, chromatin-enriched fractions of control and 6-TG/UVA treated cells. Around 100 proteins were altered by a low UVA dose to 6-TG containing cells. So far, 60 of these have been identified by Maldi-Tof MS. The analysis confirmed the modification of PCNA and identified changes in the MSH2 DNA mismatch repair factor, the Ku80 DNA binding/repair protein, and the replication-associated Mcm proteins 3, 7 & 6. The precise manner in which these proteins are modified is currently under investigation. In a parallel study, Quentin Gueranger has demonstrated extensive formation of covalent DNA protein crosslinks in cells treated with 6-TG and UVA.

Reactive oxygen species, including \(^{1}O_2\), cause DNA strand breaks which, in turn, trigger DNA damage responses. Reto Brem and Feng Li have been investigating the induction of DNA strand breaks by 6-TG/UVA treatment and the subsequent DNA damage responses (Figure 1). We previously showed that 6-TG/UVA induces DNA single strand breaks that are detectable by Comet assays. Curiously, these breaks are largely confined to the S phase of the cell cycle and little of no breakage is observed in G1 or G2 phase. Both the Chk2 and Chk1 proteins are phosphorylated indicating that the ATM- and ATR-mediated DNA damage responses are triggered by the presence of photochemically-induced DNA lesions (Figure 1b). In agreement with the activation of ATM, DNA double strand breaks are detected in some cells. The distribution of breaks suggests that these breaks are also confined to cells in S phase. Feng has also shown that the 6-TG/UVA-induced DNA photoproducts are not excised. At high levels of photochemical DNA damage, there is a reduction in the amount of the Chk1 protein that is recovered from cell extracts. This is consistent with continued turnover of Chk1 in the presence of irreparable DNA lesions although the possibility that DNA associated Chk1 may have become covalently crosslinked to DNA has not been excluded.

The vulnerability of DNA 6-TG to oxidation by reactive oxygen has clinical implications. The incidence of skin cancer is higher in patients who have been immunosuppressed with azathioprine combined with ciclosporin A. The accepted mode of action of ciclosporin is via its effect on calcineurin. Ciclosporin is also a source of reactive oxygen, however. Natalie Attard has shown that cells with defective double strand break rejoining capacity are particularly sensitive to killing by ciclosporine indicating that this drug causes significant DNA breakage. In view of the susceptibility of DNA 6-TG to conversion into irreparable DNA damage and DNA strand breaks by reactive oxygen, Natalie is investigating possible synergistic DNA damaging interactions between the two drugs.

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Publications listed on page 133
A new DNA repair-based model for the cytotoxic action of the chemotherapeutic drug, 5-fluorouracil

Excision of uracil and derivatives occurring as aberrant bases in the mammalian genome demonstrates how DNA repair can militate against malignant transformation but also modulate chemotherapy based on DNA-damaging cytotoxic drugs. Mammalian cells contain two uracil-DNA glycosylases, the ubiquitous and highly conserved Ung enzyme, and Smug, which has only evolved in insects and vertebrates. We have previously reported that both Ung and Smug suppress spontaneous C→T mutability in mammalian cells – excising uracil from U:G lesions arising by hydrolytic deamination of cytosine – whereas Ung uniquely processes U:G mispairs generated enzymatically by AID (activation induced cytosine deaminase) during antibody gene diversification in activated B cells, with Ung-deficient gene-targeted mice developing B-cell lymphomas. We have shown that it is Smug, and not Ung, which excises the fluorine-substituted uracil derivative, 5-fluorouracil (FU), incorporated in DNA, such that Smug-deficient cells are uniquely hypersensitive to this chemotherapeutic drug. FU is used in some two million patients a year, improving survival of breast, head-and-neck, aerodigestive and particularly colorectal cancer, where combination FU chemotherapy is a front-line treatment. Our data disprove a widely cited but unsubstantiated model by demonstrating that FU incorporation into DNA is a predominant cause of drug cytotoxicity. Furthermore, Smug over-expression decreases cellular FU sensitivity and up-regulation of Smug may provide a previously unrecognised mechanism of acquired drug resistance in tumours, acquired and de novo chemo-resistance being the major obstacle to successful FU-based cancer therapy. We now aim to analyse SMUG activity versus survival during FU treatment of patients and as a putative biomarker of initial drug response. These studies will be supported by micro-array gene-expression profiling of FU-responsive/resistant tumours (Elaina Collie-Duguid, Institute of Medical Sciences, University of Aberdeen), and collaborations with clinical oncologists at both Aberdeen Royal Infirmary and Mount Vernon Hospital.

A single-stranded by-product of lagging-strand DNA synthesis is degraded by the Trex1 DNA exonuclease in mammalian cells to prevent autoimmune disease

Trex1 is only found in mammals where it is the major 3’→5’ DNA exonuclease. We generated TREX1 null mice which serve as a model of the human autoinflammatory disorder, Aicardi-Goutières syndrome (AGS), a recessive genetic mimic of acquired viral infection which can be due to mutations in TREX1 or any of the three genes encoding subunits of the RNaseH2 holoenzyme. The mechanism of Trex1-deficient disease remains unclear; although a link to endogenous retroelements has been suggested. However, we have shown that Trex1, ordinarily associated with the endoplasmic reticulum, relocates to the nucleus in S phase and identify the in vivo substrate of Trex1 as a discrete 62mer
single-stranded polynucleotide by-product of DNA replication that accumulates outside the nucleus, where it could be perceived as 'foreign' and so provoke the antiviral-like autoimmunity characteristic of Trex1-deficient disease. Furthermore, we have now identified a common nucleic acid substrate for Trex1 and RNaseH2 during lagging-strand DNA replication that might underscore this genetically heterogeneous disease.

**Repair of alkylated DNA by human ABH and FTO proteins**

Methylating agents occur endogenously and in the environment, and also due to their cytotoxicity are used in cancer treatment. They methylate DNA bases generating lesions that block DNA replication or cause mutations. DNA repair activities that specifically revert some of these lesions reduce the cytotoxicity of the methylation damage. These activities are conserved from bacteria to human cells. We previously characterised *E. coli* AlkB and human ABH2 and ABH3 proteins that directly demethylate 1-methyladenine and 3-methylcytosine, major alkylation lesions that are generated in single stranded DNA. These enzymes are 2-oxoglutarate Fe2+-dependent dioxygenases that oxidise the aberrant methyl groups resulting in their destabilisation and release as formaldehyde. More recently we have identified an additional seven human homologues of AlkB (ABH1, ABH4-8 and FTO). Sequence variants in the first intron of the FTO gene have been strongly associated with obesity in several populations. In collaboration with the groups of Stephen O’Rahilly (Cambridge) and Christopher Schofield (Oxford), we have defined a biochemical activity of human FTO. The FTO protein is a dioxygenase that demethylates 3-methylthymine (but not 1-methyladenine) in single stranded DNA. 3-methylthymine is a rare DNA lesion. Using an assay that measures conversion of the cosubstrate 2-oxoglutarate to succinate, FTO was shown to specifically interact with pyrimidine nucleosides methylated at the 3’ position, including 3-methyluridine. A working hypothesis is that FTO regulates transcription or translation by demethylation of an as yet unspecified RNA. In an ongoing collaboration with Laurence Colleaux (Paris) and Stephen O’Rahilly, an Arab family carrying a mutation within the FTO coding sequence was identified. The mutated protein is inactive in our *in vitro* enzyme assays. Siblings that are homozygous for this variant have a severe developmental syndrome. These findings provide the first example of a human disorder due to a functional defect in an AlkB-related dioxygenase and reveal an unpredicted role for this protein in development.

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![Figure 1. Demethylation of 3-methylthymidine in single stranded DNA by FTO. FTO requires the cofactor Fe2+ and the co-substrates O2 and 2-oxoglutarate to catalyse hydroxylation of its substrate. The proposed intermediate degrades releasing the hydroxylated methyl group as formaldehyde and the unmodified nucleoside thymidine is directly regenerated.](image-url)
DNA stability works on many different levels: the need for the organism to survive past reproduction, the need for providing a suitable genetic pool for a species, as well as allowing for changes in the DNA to ensure adaptability. There are numerous different ways how this level of stability is achieved. Our work has focused on a class of enzymes, known as DNA deaminases. These proteins are actively recruited to DNA and deaminate cytosine to uracil. One of the best characterised DNA deaminases is AID (Activation Induced Deaminase), which is required for the maturation of antibodies during an immune response. At the genetic level deamination causes a coding change, as uracil is read as thymidine rather than cytosine. At the biochemical level, this lesion causes an alteration in the DNA structure, which in turn recruits members of the DNA repair machinery to the lesion. The outcome of the DNA deaminase induced lesion, repair, recombination, or mutation is dependent on the physiological state of the cell.

As human beings are living longer and past their reproductive age, DNA instability has begun to manifest itself in the population as cancer. Understanding how DNA instability is initiated, controlled or repaired has become the cornerstone of understanding cancer biology. Initiation of DNA instability is usually derived from exogenous sources (such as UV light or smoking) or endogenous sources (such as mistakes during DNA replication or natural oxidative damage). On the other hand, it is still not well understood how non-DNA damaging agents – such as hormones – can initiate DNA lesions that lead to cancer.

Hormonal Regulation of DNA deaminases
Because of their function and enzymatic activity, it is evident that DNA deaminases must be tightly regulated. A number of recent reports have begun to dissect how the sub-cellular distribution of AID is regulated, our own efforts have also highlighted other means by which AID is controlled. Transcriptional regulation though, is one of the more prominent pathways that we have analysed. In B cells, E-box proteins, NFκB and Pax5 have been shown to bind to the AID promoter, but it remained unclear which pathways would activate AID expression in non-lymphoid cells (such as germ cells). In our recent work, we have identified that the hormone signaling pathway can regulate AID transcription independently of other signals. Ligand bound hormone-receptors can bind directly to the AID promoter and alter AID protein production, which in turn influences downstream events such as mutation and translocation. While oestrogen will activate AID transcription, progesterone will inhibit AID mRNA formation; this antithetical regulation indicates that AID is part of normal hormonal homeostasis. While the majority of our work was done in B cells, AID production could be activated up to 25 fold in non-immune tissue, such as ovaries.

The consequence of oestrogen induced AID production in B cells is excessive antibody diversification, which may lead to autoimmunity. Interestingly, it has long been known that B cell autoimmunities such as lupus are ten times more likely in...
women then in men, and that the levels of oestrogen can alter the overall efficacy of the immune system.

**Oestrogen and DNA lesions**

The pleiotropic activities of hormones are well documented, and there is no organ or system that is not influenced by them. On the other hand, already 50 years ago it was observed that hormones such as oestrogen can enhance, if not even cause, cancer. Yet there has been very little progress in understanding how oestrogen causes DNA lesions that lead to oncogenic mutations and translocations. There is limited evidence that oestrogen metabolites can damage DNA or even induce mutations. Our finding that the DNA deaminase, which can act as a DNA mutator, is activated by oestrogen not only in B cells but in a number of different hormonally responsive tissues, proposes a new theory (Figure 1).

Here, oestrogen (even at physiological levels) will enter a cell, bind to the oestrogen receptor, transport into the nucleus, and locate to the AID promoter (or that of other DNA deaminases). This will recruit the transcription machinery, activate AID mRNA production, and initiate protein translation. This can cause misexpression of AID and allow AID to target non-physiological loci, giving rise to mutations in oncogenes and tumor suppressor genes; with some of the AID induced lesions even initiating translocations (e.g. Burkitt lymphoma, c-Myc/immunoglobulin H gene — Figure 2). Thereby, our work has shown for the first time how oestrogen can have genotoxic activity in vertebrates (only vertebrates express DNA deaminases), without causing DNA damage itself, and how oestrogen can induce DNA instability leading to cancer:

In the past, tamoxifen (a synthetic oestrogen analogue) has been used to antagonise the activity of oestrogen. In our experiments tamoxifen can inhibit the mutator activity of oestrogen, but on its own tamoxifen can also act as an agonist of the oestrogen pathway. This could explain why tamoxifen treatment can lead to secondary tumour formation; by activating DNA deaminases and thereby mutating DNA.

**Outlook**

The discovery that oestrogen can induce DNA instability, by directly activating DNA deaminases, will provide new opportunities for identifying cancer targets and preventative measures. Our current work is dissecting the molecular mechanisms of how AID induces DNA lesions within and outside the immunoglobulin locus. Proteomics and genetic screens will identify co-factors, which alter AID activity and thereby serve as targets during prolonged oestrogen treatment. This work will have a direct impact on cancer patients and their treatments.
Eukaryotic cells faithfully partition their genetic information in the form of chromosomes to daughter cells during cell division. Defects in cell division are inevitably associated with the emergence of aneuploid genomes, a feature common to most cancer cells that is characterised by the missing or supernumerary chromosomes. Given the importance of cell division for the development of malignancies, mitosis is a central target in antiproliferative cancer therapy.

Cell division involves dramatic changes in cellular architecture. After the mitotic spindle has segregated sister chromatids to opposite poles of the cell, contraction of the cell membrane separates the cytoplasm of the two nascent daughter cells. This process is referred to as cytokinesis. In animal cells, cytokinesis is accomplished by the constriction of an actomyosin-based structure, called the contractile ring. Local activation of the GTPase RhoA at the equatorial cortex in anaphase promotes the assembly of the contractile ring (Figure 1c). Following cleavage furrow ingression, membrane fusion generates two physically distinct daughter cells.

The physical separation of daughter cells during cytokinesis represents the final step of cell division and provides the basis for cell multiplication during proliferation and development. Cytokinesis also plays a key role in preventing genomic instability, a hallmark of cancer cells. Our group investigates the mechanisms that orchestrate cytokinesis in mammalian cells and explores the consequences of cell division failure and aneuploidy in the context of cancer.

In animal cells, the mitotic spindle plays an important role in positioning the cleavage plane. At anaphase an array of overlapping microtubules forms a structure referred to as the central spindle (Figure 1b and 1c). This structure is located midway between the two masses of segregated DNA and serves as a signaling platform for the initiation of cytokinesis at the overlying cell cortex. Recruitment of the conserved RhoGEF protein Ect2 to the central spindle in anaphase is thought to elicit the local activation of RhoA at the equatorial cortex, which in turn leads to the initiation of cytokinesis (Figure 1c).

Phospho-regulation of cytokinesis

The highly conserved mitotic kinase Plk1 (Polo-like kinase 1) controls a multitude of processes during cell division. Using a potent and selective Plk1 inhibitor, we have discovered a key role for Plk1 in triggering the initiation of cytokinesis in human cells. Acute inhibition of Plk1 at anaphase abolishes RhoA accumulation at the equator, contractile ring formation, and cleavage furrow ingression. We found that Plk1 regulates RhoA by promoting the interaction of the RhoGEF Ect2 with its central spindle anchor protein HsCyk-4, a subunit of the centralspindlin complex (HsCyk-4/Mklp1). Our data suggested that late mitotic Plk1 activity promotes recruitment of Ect2 to the central spindle (Figure 1c).

Over the last year we have been investigating the molecular basis for how Plk1 induces formation of the Ect2/Hs-Cyk4 complex. Using in vitro kinase assays and peptide array experiments we were able to identify a region within HsCyk-4 that contains a cluster of Plk1 phosphorylation target sites (Figure 1a). Phospho-specific antibodies raised against one of these sites confirmed that HsCyk-4 is phosphorylated by Plk1 at the central spindle during anaphase (Figure 1b). In collaboration with Michael Glotzer (University of Chicago), we demonstrated that simultaneous mutation of Plk1 target sites within HsCyk-4 blocks Ect2 recruitment to the central spindle and prevents the onset of
cytokinesis thereby mimicking the Plk1 inhibition phenotype. Ect2’s N-terminus shares homology with BRCT domains that can act as phospho-peptide binding modules. Mutation of conserved residues, which are predicted to coordinate the phosphate within Ect2’s BRCT domains, also abolishes the recruitment of Ect2 to the central spindle. In summary, our data suggest that phosphorylation of HsCyk-4 by Plk1 at the central spindle during anaphase provides a landing platform for Ect2’s BRCT domains (Figure 1c). Currently, we are exploring the dynamics and requirements of this Plk1-dependent signaling pathway that controls the activity of the key cytokinetic switch RhoA and lies at the heart of cleavage furrow induction in animal cells.

Molecular mechanisms regulating and executing cytokinesis in mammalian cells

We use a combination of mass spectrometry and functional genomics to identify molecules involved in different aspects of cytokinesis. Subsequently, we employ cell biology, biochemistry, and time-lapse microscopy to unearth the molecular function of these factors. This will help us to gain insights at the molecular level into how cells orchestrate microtubule, actin, and membrane action to bring about cell division at the right time and at the right place.

The causes and consequences of tetraploidy

A growing body of evidence links cytokinesis defects and tetraploidy to tumorigenesis. Pharmacological or genetic inhibition of cytokinesis in murine cells leads to progressive aneuploidy and tumorigenesis. Furthermore, lesions in several tumor suppressor genes perturb the successful completion of cytokinesis. These and other observations have led to the hypothesis that tetraploid cells could be a transient intermediate on the road to aneuploidy and cancer. While tetraploid cells face several challenges including a duplicated set of chromosomes and spindle poles, recent data show that polyploidy can provide an evolutionary advantage in the acquisition of new phenotypic traits.

We are using small-molecule compounds and RNA interference to generate tetraploid mammalian cells with the aim to investigate the effects of tetraploidy on proliferation, genomic stability, mitotic dynamics, and tumorigenesis (Figure 2). We plan to use our findings in cultured cells to extend our studies to animal models. Furthermore, we are interested in determining how tumor suppressor genes influence cytokinesis. Characterising the origin and fate of tetraploid cells will help us to understand the impact of cell division failure on tumorigenesis and might be useful to locate points of vulnerability of aneuploid cancer cells.

Figure 1. Phospho-regulation of cytokinesis by Polo-like kinase 1 (Plk1). a) Identification of Plk1 phosphorylation target sites within HsCyk-4 using recombinant Plk1 and a peptide array. b) Plk1-dependent phosphorylation of HsCyk-4 (Ser157) at the central spindle during anaphase. c) Model for Plk1 function in regulating Ect2 recruitment to the central spindle and triggering the initiation of cytokinesis in human cells.

Figure 2. Efficient S-phase entry and DNA replication in bi-nucleated and tetraploid human epithelial cells.
RNA polymerase II (RNAPII) transcribes all protein-encoding genes in eukaryotes and is the endpoint target for virtually all cell regulatory pathways. The overall aim of our research is to understand the basic mechanisms underlying RNAPII transcription, but in particular the mechanism and factors governing transcript elongation. For example, we strive to understand what happens when RNAPII arrests because of obstacles such as DNA damage or chromatin structure. In our work, we use a combination of biochemical and genetic approaches in yeast, Drosophila, and, increasingly, in human cells. A detailed insight into the basic mechanisms of transcript elongation will help make it possible to understand human diseases, such as cancer, and hopefully in time to learn how to treat them.

The active, elongating form of RNAPII is tightly associated with chromatin. Remarkably, although the active forms of factors involved in other DNA-related processes such as DNA replication, repair, and recombination are also associated with chromatin, proteins are rarely purified specifically from this source. We developed a protocol for the isolation of chromatin-associated factors and used it to identify proteins interacting with the actively engaged form of human RNA polymerase II (RNAPII) (Aygün et al., 2008). Surprisingly, beside a plethora of expected protein partners, one of the RNAPII-associated factors was a DNA helicase called RECQL5 (Figure 1). Members of the highly conserved RECQ family of DNA helicases play key roles in the maintenance of genome stability in all organisms examined. They are thought to act at the replication-recombination interface to suppress undesired recombination events that may occur due to stalled or damaged DNA replication forks. Loss-of-function mutation in 3 members of the human RecQ family of helicases, namely BLM, WRN and RECQ4, have been directly associated with genetic diseases that are characterized by premature aging and predisposition to various types of tumors due to increased genomic instability. We found that the RECQL5-RNAPII interaction is direct and mediated by the RPB1 subunit of RNAPII, and that RECQL5 is the only member of the human RECQ family of helicases that associates with RNAPII. The RNAPII-RECQ5 interaction represents an unexpected connection between transcription and genomic stability, possibly required for suppressing transcription-associated DNA recombination (Aygün et al., 2008). Our future work will be focused on further defining the RNAPII-RECQL5 connection and its functional importance in this and other processes.

The small protein-modifier ubiquitin is attached to innumerable target proteins. The final outcome of protein poly-ubiquitylation is often proteasome-mediated proteolysis, meaning that ‘proof-reading’ of ubiquitylation by ubiquitin proteases (UBPs) is likely to be crucial for proper regulation. Transcriptional arrest can trigger ubiquitin-mediated proteolysis of RNA polymerase II (RNAPII), so a UBP
reversing RNAPII ubiquitylation might be expected. We found that Ubp3 de-ubiquitylates RNAPII in the yeast Saccharomyces cerevisiae (Kvint et al., 2008). Indeed, genetic characterisation of cells lacking the UBP3 gene is consistent with a role for Ubp3 in transcript elongation, and Ubp3 can be purified with RNAPII, Def1, and the elongation factors Spt5 and TFIIF. This Ubp3 complex de-ubiquitylates both mono- and poly-ubiquitylated RNAPII in vitro, and cells lacking the UBP3 gene have elevated levels of ubiquitylated RNAPII in vivo. Moreover, RNAPII is degraded faster in a ubp3 mutant after UV-irradiation. We previously proposed that problems posed by damage-arrested RNAPII are resolved either by removing the damage, or degrading the polymerase. In agreement with this, cells with compromised DNA repair are better equipped to survive UV damage when UBP3 is deleted (Kvint et al., 2008 Mol Cell 30: 498-506).

Several years ago, we isolated the Elongator complex as a component of the elongating form of RNAPII. It is now known to function in diverse cellular processes, such as RNA polymerase II transcriptional elongation and tRNA modification. The Ebp3 subunit of Elongator possesses a C-terminal histone acetyltransferase (HAT) motif and an N-terminal sequence that resembles an iron-sulfur (FeS) cluster. The HAT domain is well characterised, but the role of the FeS cluster is unknown, although one report previously proposed that it might be involved in catalyzing histone demethylation (Chinenov Y. Trends Biochem Sci 2002 27: 115-117). We investigated the importance and function of the yeast Ebp3 FeS cluster by a combination of genetic and biochemical means (Greenwood et al., 2009) j. Biol Chem 281: 141-149). To minimise oxidation of the Ebp3 FeS cluster during purification, we also developed a novel tandem-affinity tag and an accompanying isolation procedure that enables purification of tagged proteins to virtual homogeneity within a few hours of cell disruption. Our results failed to support a role for Elongator in histone demethylation. Moreover, we found that FeS cluster integrity is not required for the HAT or RNA-binding activities of Elongator. However, a fully functional FeS cluster is required for Elongator integrity and for the association of the complex with its accessory factors Kti11 and Kti12. In contrast, the association of Elongator with RNAPII in chromatin is unaffected by FeS cluster mutations. Together, these data supported the idea that the Ebp3 FeS cluster is essential for normal Elongator function in vivo primarily as a structural, rather than catalytic, domain (Greenwood et al., J Biol Chem. 2008; doi:10.1074/jbc.M805312200).

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Figure 1. Mass spectrometric analysis of a human RNAPII complex purified from chromatin. a)outline of the purification procedure. B and C, equal amounts of the anti-FLAG chromatography eluates from control (Mock), nucleoplasm, b) and chromatin fractions, c) were fractionated by 4-12% SDS-PAGE and analyzed by silver staining. Asterisks indicate background protein bands that are also present in the control purification. Proteins identified by mass spectrometric analysis are labelled to the right of the respective protein bands.
SUMO modification of budding yeast PCNA in S phase

SUMO modification of budding yeast PCNA in S phase

DNA is susceptible to a variety of insults from exogenous and endogenous sources. In contrast to DNA repair systems, which usually rely on the excision and subsequent re-synthesis of the damaged region to restore the original sequence information, DNA damage tolerance mechanisms allow the bypass of lesions without their actual removal. They ensure the progression of DNA replication on damaged DNA and are therefore essential for the survival of a cell in the presence of genotoxic agents. As lesion bypass is often associated with damage-induced mutations, however, its activity needs to be tightly controlled. Our research aims at understanding the mechanisms and signals by which the ubiquitin and SUMO systems of posttranslational protein modification promote damage tolerance and limit the accumulation of unwanted mutations.

SUMO modification of budding yeast PCNA in S phase

SUMO is a posttranslational protein modifier of the family of ubiquitin-like proteins, common to all eukaryotes. Modification by SUMO has been implicated in the control of numerous biological processes, ranging from nucleo-cytoplasmic transport to the regulation of transcription, chromosome segregation and genome stability.

Our previous work has elucidated the mechanism of SUMO function in the case of budding yeast PCNA, the eukaryotic sliding clamp protein that ensures processive action of DNA polymerases during replication. We showed that PCNA sumoylation recruits an anti-recombinogenic helicase, Srs2, to replication forks by means of a preferential interaction of the helicase with the modified form of PCNA. By counteracting the formation of recombinogenic Rad51 filaments, Srs2 thus prevents unscheduled recombination events. When replication forks are stalled by DNA damage, the PCNASUMO-Srs2 system ensures a processing of the lesions via the damage bypass pathway that is triggered by the ubiquitination of PCNA.

Over the past year, our lab has investigated the architecture of the SUMO conjugates on PCNA as well as the regulation of PCNA sumoylation throughout the cell cycle. Purification of the SUMO conjugation machinery has allowed us to reconstitute the reaction in vitro and to complement our in vivo analysis with mechanistic insight into the modification reaction.

Architecture and assembly of poly-SUMO chains on budding yeast PCNA

Like other members of the ubiquitin family, SUMO is covalently attached to its target proteins by an enzymatic machinery comprising an activating enzyme (E1), a conjugating enzyme (E2) and a ligase (E3). The single E2, Ubc9, exhibits a marked preference for a short consensus motif, ΨKXE (where Ψ denotes a hydrophobic and X any amino acid). The predominant site of PCNA modification, K164, does not obey the consensus motif, despite being highly conserved among eukaryotic PCNA sequences. K127, which conforms to the consensus, is a minor site of attachment that is predominantly used when K164 is mutated. The ligase responsible for modification is Siz1, and SUMO is removed by the isopeptidase Ulp1.
We have analyzed the SUMO modification pattern of budding yeast PCNA and found that most aspects of our in vitro sumoylation reactions reflect the situation under physiological conditions. Two oligomeric SUMO chains of two to three moieties each, linked via internal sumoylation consensus motifs within the SUMO sequence, are assembled on PCNA. Siz1 both stimulates the overall efficiency of sumoylation and selects the modification site on PCNA. Furthermore, ubiquitin and SUMO chains are assembled independently, and we found evidence that both modifiers can coexist in vivo on a common PCNA subunit. Our results demonstrate for the first time the in vivo assembly of polymeric SUMO chains of defined linkage on a physiological substrate in yeast, but they also indicate that SUMO–SUMO polymers are dispensable for PCNA-SUMO function in replication and recombination.

**Activation of S phase-dependent PCNA sumoylation by DNA**

Modification by SUMO is often regulated by cellular signals that restrict the modification to appropriate situations. Nevertheless, most SUMO-specific ligases do not exhibit much target specificity, and – compared with the diversity of sumoylation substrates – their number is limited. This raises the question of how SUMO conjugation is controlled in vivo. We have now discovered an unexpected mechanism by which sumoylation of budding yeast PCNA is effectively coupled to S phase. We found that loading of PCNA onto DNA is both necessary and sufficient for sumoylation in vivo and greatly stimulates modification in vitro. Given that Siz1 contains a SAP domain, which in many other proteins mediates DNA binding, we argued that DNA would likely act as a template for bringing the E3 and the substrate into close proximity, thus enhancing the efficiency of the reaction. In fact, we were able to show that Siz1 binds to double-stranded DNA by means of its SAP domain. To our surprise, however; DNA binding by Siz1 was not strictly required for efficient PCNA sumoylation. Instead, we found that the stimulatory effect of DNA on conjugation is mainly attributable to the loading of PCNA itself. These findings imply a change in the properties of PCNA upon DNA binding that enhances its capacity to be sumoylated, and we have indeed identified mutations in the inner, DNA-contacting surface of PCNA that severely impair its capacity to be sumoylated. Hence, the loading of PCNA onto DNA during S phase provides a simple, yet effective mechanism to limit sumoylation to the relevant cell cycle stage (Figure 1).

**Publications listed on page 133**
Our DNA is continually subjected to damage, either from endogenous sources such as reactive oxygen species that are produced as by-products of oxidative metabolism, from the breakdown of replication forks during normal cell growth, or by agents in the environment such as ionising radiation or carcinogenic chemicals. Fortunately, cells have evolved to cope with damage by employing elaborate and effective repair processes that are specialised to recognise certain lesions in DNA and to repair them.

DNA double-strand breaks represent one of the more dangerous forms of damage, as they can lead to aberrant gene translocations. The consequences of aberrant repair can be catastrophic to the cell and lead to cancer. One major pathway of double-strand break repair involves the enzymes of genetic recombination. Although genetic recombination normally occurs in germ-line cells at meiosis, where it provides a mechanism for the exchange and reassortment of genetic information, it also plays a critically important role in somatic cells for the repair of damaged or broken chromosomes. Our interest in the contribution of genetic recombination to the repair of DNA double-strand breaks stems from observations indicating that cell lines derived from individuals predisposed to breast cancer through mutations in \textit{BRCA2} exhibit a genome instability phenotype characteristic of a recombination/repair defect.

Recombinational repair and breast cancer

The importance of gaining a thorough understanding of homologous recombination is highlighted by observations indicating that individuals with mutations in \textit{BRCA2} have an extremely high probability (70% during their lifetime) of developing breast or ovarian cancers. The process of homologous recombination (HR) requires a number of proteins including RAD51, RAD52, RAD54, the RAD51 ‘paralogs’ (RAD51B, RAD51C, RAD51D, XRCC2, XRCC3), BRCA2 and RP-A. Many of these proteins have been purified in this laboratory, and we use biochemical, cytological and molecular biological approaches to understand how they function within the cell to repair DNA breaks (McIlwraith et al., Molecular Cell 2008; 32: 313).

Of particular importance for HR is RAD51, a protein that catalyses the key reactions required for DNA pairing and strand exchange. In response to DNA damage RAD51 localises to distinct sub-nuclear assemblies (foci) where the repair reactions take place. The localisation of RAD51 to repair foci is dependent upon the breast cancer-associated tumour suppressor BRCA2. It is now clear that BRCA2 controls RAD51 activity throughout the cell cycle and in response to DNA damage. The two proteins interact directly.
mainly through the 8 BRC regions of BRCA2 that map within exon 11, but also at an unrelated site close to the C-terminus of BRCA2. The C-terminal interaction domain is thought to supply regulatory functions, through phosphorylation at S3291, whereas the BRC repeats represent a scaffold for multiple RAD51 binding and relocalisation.

Much of our understanding of the molecular actions of BRCA2 comes from the study of small fragments of the protein. However, we were recently successful in purifying a BRCA2-RAD51 complex from human cells grown in culture and this holds great promise for future biochemical studies. We also achieved a long-term goal, in that we were successful in identifying the human Holliday junction resolvase as GEN1 (Ip et al., Nature 2008; 456: 357). The resolvase was identified using a two pronged approach: GEN1 was identified by mass spectrometry following extensive fractionation of HeLa cell-free extracts, whereas its yeast counterpart (Yen1) was detected using a TAP-fusion library screen for nucleases capable of Holliday junction resolution. Although these resolvases catalyse Holliday junction cleavage by a mechanism analogous to that shown by the E. coli Holliday junction resolvase RuvC, they represent a new sub-class of the Rad2/XPG family of structure-specific nucleases. Preliminary indications are that, like BRCA2, GEN1 deletions/frameshift mutations may be linked to increased risk of breast cancer.

Crosslink repair and Fanconi anemia

Fanconi anemia (FA) is a rare autosomal disorder characterised by congenital abnormalities, bone marrow failure and increased incidence of cancer. Cells derived from individuals with FA exhibit a chromosome instability phenotype and are hypersensitive to agents that form DNA crosslinks. These cell lines can be classified into 12 complementation groups, and the genes responsible have now been identified. One of these genes is FANCM, which encodes a putative DNA helicase that is thought to be involved in the recognition of crosslinks as they impede the progress of the DNA replication apparatus. Our biochemical analysis of FANCM is continuing with particular emphasis on defining its interaction partners.

Defective DNA repair and neurological disorders

For some time we have been interested in how defects in some DNA repair processes are associated with neurological disorders (Rass et al., Cell 2007; 130: 991). We have been successful in defining the molecular defect associated with a disease known as Ataxia with Oculomotor Apraxia1 (AOA1), which is due to defects in a protein known as Aprataxin. The biochemical properties of Aprataxin suggest that it acts as a proofreader for DNA ligases (Ahel et al., Nature 2006; 443: 713 and Rass et al., J. Biol. Chem. 2008; 283: 33994), indicating that the neurological problems associated with AOA1 are caused by the progressive accumulation of persistent nicks that cannot be repaired when Aprataxin is inactive.

Our studies of Aprataxin led us to note that a repair protein known as APLF (Aprataxin-PNK-Like Factor), whose precise cellular function is unknown, contains a novel form of zinc finger that binds to poly(ADP-ribose). Remarkably, these zinc fingers are found in a number of proteins associated with the DNA damage response and checkpoint regulation (Ahel et al., Nature 2006; 451: 81). These studies provided the first example of a zinc finger motif that interacts with poly(ADP-ribose), often considered to be the third form of nucleic acid.

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The replication and repair of DNA requires a vast array of different proteins. In order to understand what goes wrong when DNA is damaged or replicated incorrectly, we have been studying a number of systems to learn more about the details of these processes at the molecular level. The systems we work on are often bacterial as these model systems can help us to understand the basic mechanisms utilised by these enzymes but the proteins are often much more amenable to structural analysis than their human equivalents yet operate by similar mechanisms.

Mechanism of DNA helicases involved in repair of DNA
In bacteria, chromosome breaks are processed by the RecBCD enzyme complex. RecBCD acts by binding tightly to the blunt end and then, by using two helicase motor subunits of opposite polarity, it unwinds the DNA. The two separated strands are both digested by a single nuclease domain in the RecB protein. This process of DNA degradation continues until the enzyme encounters a specific eight base sequence known as a Chi site, at which point the activities of the enzyme change. The nuclease activity is attenuated on one strand but continues on the other to produce a 3’ tail, onto which RecBCD loads the RecA protein. RecA then initiates a homologous recombination reaction to re-establish a replication fork and thus repair the break.

RecB and RecD helicase subunits have opposite polarities of unwinding. In order to understand the molecular determinants of helicase directionality we have determined the crystal structure of complexes of RecD with ssDNA in the presence and absence of an ATP analogue, ADPNP (Figure 1). These structures reveal how the enzyme is able to walk along DNA in a 5’-3’ direction in an ATP-dependent manner. In collaboration with Martin Webb’s group at NIMR, London we have shown that the step size for translocation along DNA is one base per ATP hydrolysed, consistent with the mechanism suggested by the crystal structures.

Another area of active investigation is the interaction between the RecBCD complex and RecA in order for us to understand the mechanism by which RecA is loaded onto DNA as a prelude to homologous recombination. Recently, in collaboration with Ed Egelman at the University of Virginia, we have obtained EM data showing how the nuclease domain of RecB interacts with RecA filaments.

During DNA replication, the progression of the replication fork frequently stalls due to DNA damage that is detected by the polymerase during chain extension. Stalling of the replication fork is followed by disassembly of the replication apparatus. There appear to be several ways that stalled forks can be processed. One system that we have been studying involves reversal of the replication fork by a protein called RecG. This protein ‘backs up’ the fork to form a four-way ‘Holliday’ junction. This process allows the DNA polymerase to use a different DNA strand as the template for replication in a process called ‘template switching’. Once the chain has been extended further, the four-way junction can be
regressed past the original DNA lesion and replication can proceed. By utilising this mechanism, the DNA damage is by-passed but maintains the fidelity of replication. The DNA damage can then be repaired at a later stage.

In order to understand how RecG catalyses DNA replication fork reversal, we have determined the X-ray crystal structure of the enzyme bound to a synthetic DNA substrate that mimics a stalled replication fork. However, the substrate used previously was rather too short to extend across the motor domains and now we have crystallised the protein with longer DNA substrates that reach across to these domains. We have also crystallised the complex in the presence of an non-hydrolysable ATP analogue to determine the mechanism by which RecG walks along double-stranded DNA.

A molecular switch in AAA+ proteins
AAA+ proteins are involved in many processes in cells including DNA replication and repair. The ATPase activity of these proteins is regulated by ligand binding and also by association with other protein co-factors. However, the mechanism of this control was unclear. In a collaboration with Xiaodong Zhang at Imperial College London, we have identified a mechanism for this process. Analysis of structures in the protein database show that an active site glutamate residue adopts two different conformations in these proteins, one of which is competent to promote hydrolysis of ATP while the other is not. Ligand binding switches the glutamate residue between these two conformations and hence regulates ATPase activity. This switch was first observed in a transcriptional activator by Prof. Zhang’s group. However, our work has extended these observations to look at different states of the proteins from different AAA+ families. Structures of an archael replication initiator protein bound to DNA that we reported last year show how this switch operates in these proteins to prevent ATP hydrolysis when the protein is bound to DNA. Similarly, our previous work on structures of the eukaryotic DNA polymerase processivity clamp (PCNA) loading complex (Replication Factor C) combined with biochemical data on mutant enzymes show that this regulation process works in the opposite direction with DNA binding being the cause of stimulation of the ATPase activity. Consequently, the switch operates across many different AAA+ families and reveals a regulatory mechanism that is widespread in AAA+ proteins.

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Figure 1. Crystal structures of the complexes of D.radiodurans RecD2 with single stranded DNA in a) the absence and b) presence of an ATP analogue
The London Research Institute benefits from access to a wide range of high quality research services. In the past, scientific support for researchers at LRI has been provided by eight core facility labs run by the Institute itself, and also by the Cancer Research UK Research Services Directorate, which in addition served researchers in the wider Cancer Research UK community. Following an external review of the Cancer Research UK Research Services Directorate in March 2008, those core facilities where LRI researchers make up the majority of the users will now been brought under direct LRI management.

Technology core facilities provided centrally at LRI allow access to state of the art equipment and instruction in its correct and effective usage. Within these facilities, services may either be run by dedicated staff or by researchers themselves with appropriate service staff input. LRI services include the following: Light Microscopy provides conventional, confocal and automated microscopy, time-lapse video and microinjection services. Electron Microscopy has a field emission scanning electron microscope, transmission electron microscopes with tomography and cryo EM capability, plus a high-pressure freezer. The FACS laboratory provides a comprehensive and continuously evolving flow cytometry service. The Experimental Pathology laboratory analyses the phenotypes of transgenic mice as well as providing expertise in human histopathology. The In Situ Hybridisation service allows visualisation of mRNA levels in tissue samples. The Protein Analysis laboratories at Lincoln’s Inn Fields and Clare Hall provide mass spectroscopy using a total of five LC-MS systems, plus two-dimensional gel electrophoresis technology. The Equipment Park provides DNA sequencing, robotic nucleic acid preparation, quantitative PCR, gel imaging systems and HPLC micro-purification, as well as automation for high throughput screening. Finally, the high throughput screening facility, set up in 2007 under Mike Howell at Lincoln’s Inn Fields, brings together the equipment, personnel and expertise needed to carry out and interpret large scale screening assays, such as cell-based screening of genome-wide human and Drosophila RNA interference libraries held by the institute. The lab is equipped with robotic systems, automated plate readers and automated high content microscopy systems.

The quality and development of LRI core facilities is driven by user committees, made up of representatives of the service provider, users and management which provide advice on technical advances, prioritise projects when facilities are limited, and act as a focal point for interactions with researchers. In addition, a programme of review by external experts every three years ensures that LRI services remain cutting edge. Major developments in LRI services over the past year have seen the incorporation of two new core facilities taken over from CRUK Research Services. The Protein Production lab under Sven Kjaer specialises in the production of pure recombinant proteins for structural studies, using baculoviral, bacterial and mammalian tissue culture systems. The Bioinformatics and Biostatistics unit provides support for all the institute’s bioinformatics needs, ranging from high throughput sequencing data analysis and high throughput screen interpretation to global gene expression analysis. It is led by Aengus Stewart with a staff of eight.

In the coming year, further core facilities from CRUK Research Services will be incorporated into the LRI, including Cell Services, Fermentation Service, Histology Service, Peptide Synthesis and Transgenic Service. LRI researchers will continue to use the CRUK Paterson Institute’s Microarray Service for gene expression profiling, including exon arrays, and also SNP genotyping and copy number analysis, and high-density tiling arrays. In addition, LRI researchers have been the major users of the CRUK high throughput sequencing facility in Leeds and the institute is also now in the process of establishing its own high throughput sequencing facility at Lincoln’s Inn Fields.
High-throughput technologies have become standard tools for answering complex and large biological questions as high density arrays and next-gen sequencers are now routinely used to address questions involving DNA binding proteins, epigenetic studies, large-scale SNP detection and chromosomal structural rearrangements, many on a genome wide scale.

All of these generate very large datasets and the methodologies and techniques of bioinformatics are intrinsic to their analysis and interpretation.

The Bioinformatics and Biostatistics service in addition to supporting high throughput technologies provides support for sequence analysis, genomics, and mathematical and statistical approaches to biological data. This is provided through expert consultation and collaboration, the development of software for analysis and visualisation, and training courses.

miRNA expression profiles using Next-Generation Sequencers
We have developed an analysis pipeline for miRNAs using data generated from the Illumina Genome Analyzer sequencer. The raw sequence reads are screened for quality and adapter trimmed, then sorted and counted into unique sequence tags. These tags are screened against a series of databases to identify known miRNAs, miRNA hairpins, and other RNA classes. The counts of known small RNAs can then be compiled and analysed across samples to generate discriminatory expression profiles. Potentially novel miRNAs can be suggested by screening high count unidentified tags against the genome and investigating the structural integrity of the predicted miRNA precursor. Candidates can then be biologically verified.

Identification of splicing events
Working with the Translational Cancer Therapeutics laboratory we identified gene expression changes and alternative splicing events associated with cytotoxic drug treatment and CERT depletion in HCT-116 cells using the Affymetrix exon array platform. We developed analysis software to process the data at the gene and exon level along with infrastructure to handle annotation. We demonstrated that gene expression changes as measured by the exon array correlated well with Quantitative RT-PCR results. Work continues to validate putative splicing events using a custom long oligo array.

Development of a novel gene interaction network
In order to separate potential candidate tumour suppressor genes from genes up-regulated due to stress response in an Emetine treatment experiment we developed a novel gene interaction network. A ‘complete’ human protein interactome was created using a unified view of data from the BioGRID, HPRD and BIND databases within Cytoscape, an open source bioinformatics tool for visualising molecular interaction networks and integrating gene expression profiles. By scanning sub-networks, candidate TSGs that had multiple interactions with known cancer associated genes and had few connections to stress responsive genes were prioritised for further investigation.

Figure 1 Chip-seq data for SRF binding at egr1 site
The EM Unit has experience in processing samples as diverse as proteins, viruses, yeast, cell cultures, tissues, Drosophila, Caenorhabditis elegans and zebrafish. We utilise a wide range of sample preparation techniques including:

- Semithin and ultrathin sectioning
- Cryosectioning and immunolabelling for protein localisation (TEM)
- Immunolabelling (SEM)
- High pressure freezing and freeze substitution
- Whole mounts
- Freeze fracture
- Negative stain

We have a range of electron microscopes including:

- FEI Tecnai G2 Spirit Biotwin 120kV TEM optimised for tomography
- FEI Tecnai G2 Spirit Twin 120kV TEM optimised for cryoEM
- Jeol 1010 100kV TEM
- Jeol 6700F field emission SEM

Sample preparation equipment includes:

- Three Leica ultramicrotomes for room temperature sectioning
- Two Leica cryoultramicrotomes for cryosectioning
- Leica EMPACT high pressure freezer and freeze substitution units
- FEI Vitrobot plunge freezer
- Emitech freeze dryer and Polaron critical point driers
- BalTEC, Polaron and Gatan high resolution coaters
- High specification workstation for 3D reconstruction of tomography data sets

We continue to introduce and develop new EM techniques and technologies in response to the samples and scientific questions received from the LRI research groups, concentrating on:

- Correlative Light-Electron Microscopy (Figure 1)
- Cryo techniques including CEMOVIS, cryoTEM and cryoSEM
- 3D EM including tomography and FIB/SEM
- Xray techniques including microCT and nanoCT

The EM unit is involved in teaching students and research scientists the principles and practice of electron microscopy. In addition we organise a bimonthly London EM Users group, and the EM team regularly attend microscopy courses and conferences in order to stay at the forefront of our specialisation.

For further details please see the EM unit website or visit the department for advice on electron microscopy and to discuss current and future projects.

**Publications listed on page 134**
The Equipment Park provides access to state of the art molecular biology instrumentation and offers instruction in the correct and efficient use of the technologies involved. The range of equipment is constantly reviewed and specific requests from research laboratory heads are encouraged.

The technologies include:

**Applied Biosystems 3730 DNA Analyzer** – the latest generation of capillary sequencing platforms, providing high-throughput, improved data quality and significant reduction in costs. Sequencing protocols, reaction mixes, QC samples and trouble-shooting advice are available.

**Qiagen BioRobots 8000** – we provide a high throughput nucleic acid extraction, purification and quantification service.

**Molecular Dynamics Phosphorimagers** – the STORM imaging system is an optical scanner that produces digital images of isotope-labelled samples. Phosphor screen autoradiography offers many advantages over traditional film autoradiography.

**Li-Cor Odyssey Infrared Imager** – the imaging system offers a different way to analyse blots and gels. Odyssey is uniquely equipped with two infrared channels for direct fluorescence detection enabling simultaneous probing of two separate targets on the same gel, e.g. Western blots.

**Real-Time PCR quantitation** – we have four systems designed to detect fluorescence during the thermal cycling of PCR. By plotting the increase in fluorescence versus the cycle number, the system produces amplification plots that provide a more complete picture of the PCR. The latest system allows the use of high-throughput micro-fluidic card for gene expression studies. Reagents and consumables are available from the Equipment Park.

**Agilent 2100 Bioanalyzer** – the instrument detects biomolecules by laser-induced fluorescence. During chip preparation, a dye concentrate is mixed with the gel. With the help of the priming station, the channels of the chip are filled with the gel-dye mix. During the chip run, the dye intercalates directly with DNA or RNA.

**Biomek FX Robot** – advanced liquid-handling, providing PCR and DNA sequencing reaction set-up and processing in 96 and 384 well format.

**PerkinElmer EnVision Multilabel Reader** – the instrument features modular label-specific optical mirror modules, high energy flash lamps, high-speed detectors and can accept micro-plates from 96 to 1536 wells. EnVision can handle kinetic measurements for enzyme assays and scanning of the well area for cellular assays. Detection options include Fluorescence Intensity, Fluorescence Polarisation, Time-Resolved Fluorescence, Luminescence and Absorbance.

**Beckman GenomeLab GeXP** – the system employs a universal priming strategy for gene expression analysis by which up to 30 genes can be multiplexed in the same reaction.

Figure 1. Fluorescent apoptosis assay data generated on the PerkinElmer EnVision plate reader.
Experimental Pathology offers expert help in a range of techniques for the analysis of cells and tissues from experimental animals and human bioresources. Advice, training and expertise in microscopy, dissection and imaging are provided to scientists at the LRI, with the ultimate aim of relating pathology of mouse models of disease to that of human diseases.

In the last year our image capture/analysis software platform was upgraded to NIS Elements; the user-friendly interface and sophistication allows novice users to be instructed in taking publication standard microscopic (including fluorescence multi-channel) images.

**Multichannel image**

NIS Elements also offers a flexible range of measurement parameters, such as taxonomic labelling, cell counts, lengths and areas. Measurements can be made by drawing directly onto the image, with output results being exported to any spreadsheet editor. The automatic function allows measurement of 90 different object/field features and image pixels can be segmented according to user-defined classes based on intensity, RGB and HIS values. Object counting is possible using thresholding and restrictions and the rich spectrum of mathematical morphology filters for object classification can be used to segment binary and greyscale images for measurement.

Continued improvement of our adult mouse tissue dissection and handling protocol, coupled with improvements in fixation technique, including perfusion fixation will provide optimal preservation and analysis of subtle tissue changes attributable to aberrant gene expression.

**Summary of other technical services**

- **Embryonic Analysis** – Expertise in normal and pathological development
- **Histological sectioning/staining of frozen/fixed tissue** – Experience of diverse samples (Drosophila embryos, cell lines, embryos E7.5-E19, adult mice organs).
- **Dissecting (photo) microscopy, Macro-photography and High resolution microscopy**
- **Mouse specific IHC** – Panel >170 antibodies, standard set of control and tumour tissue for optimising antibodies. 16000 automated stainer for high throughput.
- **Microscopic Histopathological analysis** – Identification of strain versus mutation specific changes, specialist interpretation of tissue structure with human comparative pathology.
- **Whole-mount organ staining/analysis** – Mammary gland (Carmine), β-galactosidase and skeletal staining, X-raying.
- **Whole-mount in situ hybridisation (ISH)**
- **3D reconstruction** – of serially stained sections for a 3-D view of cellular relationships/tissue architecture.
- **Laser Capture Microdissection** – for DNA/RNA extraction (from frozen /FFPE tissues)

We encourage close interaction with users and scientists are encouraged to visit the department for advice and to discuss current and future projects. Teaching is also an essential activity of the lab; we provide individual training in imaging, tissue handling, IHC, and other laboratory techniques as well as microscopy reviews and discussion. Further information is available on the EPL internet pages.

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Fluorescence Activated Cell Sorter
www.london-research-institute.org.uk/facs

Head Derek Davies

Staff
Kirsty Allen Ayad Eddaoudi Dolores Martinez Garcia
Laura Bazley Tina Luke Sukhveer Purewal

The FACS laboratory at the London Research Institute is a dedicated service offering a comprehensive flow cytometry analysis and sorting facility. Flow cytometry is a sophisticated form of quantitative fluorescence microscopy where cells in suspension are passed through a laser excitation source and emitted fluorescence can be detected and measured. Any part or function of a cell that can be identified with a fluorochrome may be measured by flow cytometry.

Analytical cytometers
We have 6 analytical cytometers including one plate-based bead reader and one static cytometer. These are all able to be user-operated and we offer a one to one training for all new users of the facility on any of these.

Two FACS Caliburs – 4 colours, dual laser (Blue and red)
LSRII-A – 13 colours 4 lasers (UV, violet, blue and red)
LSRII-B – 16 colours, 4 lasers (Violet, blue, yellow and red)
FACS Array – 4 colours, 2 lasers (Green and red)
Laser Scanning Cytometer – 6 colours, 2 lasers (Blue and red)

Cell sorters
We also have three cell sorters that are all able to retrieve up to 4 specifically defined populations so that cells may be recovered for further study including re-culture, RNA or DNA extraction or use in functional cell assays. These are all operated by the staff of the Laboratory but we do this in close consultation with our users.

MoFlo 1: 9 colours, 3 lasers (UV, blue and yellow or red)
MoFlo 2: 9 colours, 3 lasers (UV, blue and red)
FACS Aria – 12 colours, 3 lasers (Violet, blue and red)

Other services
We are available to advise on a wide variety of cytometry related subjects including design of experiments, sourcing and supplying reagents, data analysis and interpretation as well as data presentation. The latter is particularly important as journal requirements for flow cytometric data are becoming more specific and demanding.

Technical developments
To move the service forward we introduce methods that would be useful to our users – either new techniques not currently available or by improving current techniques. In the past year for example, we have introduced dyes that are used to identify dead cells after fixation and have improved detection of cell proliferation in fluorescent protein expressing cells by means of an EdU proliferation method.

We encourage close interaction with users of the facility and will collaborate on specific projects that need cytometric input and expertise.

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MoFlo 2: 9 colours, 3 lasers (UV, blue and red)
FACS Aria – 12 colours, 3 lasers (Violet, blue and red)
This year marked the first full year of operation for the High-Throughput screening facility. In that time we have completed four genome-wide siRNA screens and assisted on a number of other smaller projects and assay development programmes. In addition we have continued to develop the facility by improving our instrument capabilities and expanding our range of services.

**RNAi and siRNA screens**
This year we have completed four genome-wide screens with LRI research groups. We have collaborated with:

- The Secretory pathways laboratory on an siRNA screen for regulators of autophagy in mammalian cells
- The Apoptosis and Proliferation Control laboratory to look for regulators of the Hippo signalling pathway in Drosophila cells
- The Transcription laboratory to screen for regulators of the Serum response pathway in both Drosophila and mammalian cells.

We have completed both primary screen and secondary screens for these projects and will soon be validating selected hits.

**Chemical and yeast screens**
This year we have expanded our range of services by making available a number of collections of well-characterised, small molecule inhibitors from Calbiochem (kinase inhibitor collections) and Sigma (LOPAC 1280). Such collections have proved very popular and serve as both a tool for validating siRNA screening data and as a discovery tool when siRNA transfection is not possible. In addition we have begun to integrate yeast genetic screens into our portfolio of services with the acquisition of a colony picking and plating robot (Genetix, QPixXT). This will enable us to perform a variety of screens using widely available *S. Pombe* and *S. cerevisiae* viable deletion libraries.

**Infrastructure**
In addition to the QPixXT, we have acquired an Acumen Explorer eX3 laser scanning cytometer to enable fast, multi-channel, object-based fluorescent intensity measurements on adherent cells such as is needed for cell cycle analysis. This year has also seen the facility begin to fulfil its ambition to provide a central information resource for LRI groups with the launch of our intranet website. In addition we have developed a database to manage screen data and enable it to be viewed and browsed by all LRI research groups.

**Future directions**
We are currently developing projects with a number of other LRI research groups and expect to perform 5-6 genome-wide siRNA screens next year. Where possible we will increase the range of reagents and services provided and expect that with our new equipment will be able to develop novel and technically challenging screens.

**Publications listed on page 135**
This Service exists principally to help researchers localise the sites of expression of specific mRNAs in histological sections of normal or diseased tissue of any species.

The Service has significant expertise, having hybridised over 82,000 sections of tissues that have been fixed and embedded routinely, with a very high rate of success for detection of mRNAs using riboprobes labelled with $^{35}$S (or $^{3}$H for higher spatial resolution). We find isotopic probes are usually informative at the first attempt, provided that probe and tissue quality are adequate, although we have continued to explore alternative techniques for the demonstration of miRNA.

Increasingly we are called on to use genome- or chromosome-specific probes to establish the origin of cells in tumour xenografts or after cell transplantation, or in chimaeras generated by blastocyst fusion. We are able to combine immunohistochemistry or cytogenetic ISH with detection of an abundant mRNA using non-isotopic in situ hybridisation and multichannel imaging (Figure 1).

Our results allow researchers to identify the cell type or types in which a gene is expressed, and in what circumstances. In situ analysis can make an important contribution to validation of other data, e.g. [Armstrong et al., Arterioscler Thromb Vasc Biol. 2008 Sep;28(9):1640-6].

Semi-quantitative scoring can reveal whether the level of expression is related to tumour development or treatment in pre-clinical models, e.g. we have used in situ hybridisation for VEGFR2 mRNA and detected altered expression in endothelial cells following treatment of adenomas with a tyrosine kinase inhibitor [Alferez et al., Mol Cancer Ther. 2008 Mar;7(3):590-8].

One of the greatest advantages of in situ hybridisation is that patterns of expression of several genes can be compared and contrasted with each other using just a few sections of tissues that can be in very short supply, whether relatively rare clinical specimens (e.g. [Jones et al.,] Pathology 2008;216: 408-417), or very small early lesions in mouse cancer models (e.g. [Segditsas et al., Hum Mol Genet. 2008 17(24):3864-75]).

A User Committee considers applications for projects or training made by researchers from the London Research Institute and other CRUK laboratories so that we undertake around 25 projects each year.

Our intranet pages show examples of results and offer advice on how to design probes so that they are specific.

The ISH Service team is always pleased to help researchers with their project.

Publications listed on page 135

Figure 1. Combined detection of PODXL mRNA and both X and Y chromosomes to determine the origin of podocytes in a female mouse following male bone marrow transplantation; a) monochrome transmitted light image showing formazan-detected mRNA in the cytoplasm of podocytes (P) on the periphery of a renal glomerulus; b) plus image planes showing FISH signals for X (red) and Y (green) chromosomes and DNA (DAPI = blue); c) false colouring of panel a in orange to enhance visibility of mRNA signal reveals the central podocyte appears to be of bone marrow origin.
Available imaging technology
Confocal microscopes LSM 510 (Zeiss) and SP5 (Leica) including a multiphoton system with tunable Chameleon laser (Coherent), UltraVIEW confocal imaging systems (PerkinElmer), low light level imaging systems (Molecular Devices and Kinetic Imaging) including high content screening and Total Internal Reflection Fluorescence (TIRF) microscopy (Nikon), video microscopes (Olympus), and microinjection systems (Eppendorf) have been configured for contrast enhancement, high resolution 3D, spectral and dynamic imaging of cells in multiple fluorescence channels with optical sectioning using motorised focus at multiple fields. Image processing and statistical analysis can be employed for deconvolution, colocalisation, automatic or interactive segmentation of cells and intracellular structures, morphometry, and tracking using Huygens (SVI), Velocity (Improvision), Imaris (Bitplane), AQM (Kinetic Imaging), Metamorph (Universal Imaging), MATLAB (MathWorks) and Mathematica (Wolfram Research).

Research applications examples
We developed a novel direct viewing cancer cell invasion assay with shear flow in vitro using analysis of high-resolution images, illustrated in Figure 1. This assay involves a custom-made flow chamber; specially developed cell labelling, observation by inverted wide-field microscopy and image processing-based quantitation of cell invasion. We applied this assay to metastatic sarcoma cells, where the cells invaded monolayers of endothelial cells. The metastatic sarcoma cells were labelled with green Vybrant DiO (Invitrogen) and the endothelial cells were labelled with CellTracker Orange CMTMR (Invitrogen) using a modified protocol (100 mM CMTMR in DMSO was incubated at -20°C for at least 2 weeks prior to use). Our findings showed that after adhesion, the cells initially invaded significantly faster under flow conditions compared to situations without shear stress. Later, however, the rate of invasion under flow decreased and the metastatic cells without shear stress achieved significantly higher levels of invasion. Our observations thus revealed the non-linear modulation of a tumour cell invasion process by shear flow, demonstrating that tumour cells can respond to flow by enhancement of invasiveness similarly to white blood cells (Hagglund et al., Frontiers in Bioscience; in press). We also collaborated in a project with the Leukocyte Adhesion laboratory (Nancy Hogg) where we analysed migration of T cells by Interference Reflection Microscopy and identified different patterns of cell adhesion determined by specific epitopes (Stanley et al., Embo J 2008; 27: 62-75).

Publications listed on page 136
Proteomics is the study of the protein complement of a biological system at a specific time and under specific conditions. In a broader sense, it can also be considered as the analysis of protein modifications, cellular and sub-cellular localisations and protein-protein interactions within complexes. The Protein Analysis & Proteomics laboratory at Clare Hall implement state of the art mass spectrometric techniques, enabling the identification and characterisation of proteins.

**Qualitative analysis**

The principal approach is 'bottom-up' proteomics, where proteins are enzymatically digested with proteases producing a peptide pool representative of the original proteins. Identifications from these complex mixtures are performed using nano-scale capillary LC gradient separations coupled to either a hybrid Q-ToF mass spectrometer (Waters SYNAPT HDMS) or linear ion trap mass spectrometer (ThermoFisher LTQ XL). Peptide sequences and hence the proteins from which they were derived are elucidated using the MASCOT database search engine to interrogate the UniProt, or in-house protein sequence databases.

**Characterisation of Post Translational Modifications**

Post Translational Modification (PTM) of proteins plays a fundamental role in the control of a variety of biological functions and activities. The ThermoFisher LTQ XL-ETD (Electron Transfer Dissociation), which uses ion/ion chemistry to provide sequence information, allows us to accurately assign sites of PTM such as phosphorylation and acetylation.

**Quantification**

Relative protein quantification can be performed by Stable Isotope Labelling by Amino acids in cell Culture (SILAC) which relies on metabolic incorporation of the quantitative labels, such as $^{13}$C$_6$ lysine and arginine. The extent of incorporation approaches 100%, so there are no labeling efficiency differences between samples and proteins are uniformly labeled, allowing several peptides from the same protein to be compared. During 2008 we have collaborated with Matrix Science on the development of Mascot Distiller Quantification Toolbox for quantitative mass spectrometric experiments.

**Collaborative Research**


**Publications listed on page 136**
The Protein Analysis laboratory provides services in 2D-gel separation and Fluorescence 2D difference gel electro-phoresis (2D-DIGE) as well as protein identification and characterisation by mass spectrometry using a range of methodologies and instrumentation listed below.

**Instruments**
- Agilent 6510 Q-TOF Mass spectrometer
- Applied Biosystems 4700 TOF-TOF Mass spectrometer.
- Applied Biosystems Q-Trap 4000 Mass spectrometer
- 3x Agilent 1200 Nano-LC systems.
- GE Healthcare Ettan DIGE Imaging System

**Peptide Sequencing Nano-LC MS/MS:** In-gel digests of bands from 1D gels or 2D spots are subjected to analysis by nano-LC MS/MS. Peptides separated by reverse phase LC are sequenced by low energy collision induced fragmentation (CID) using a Q-TOF mass spec employing chip based nano-LC technology. MS/MS data is automatically searched against updated databases. Users now have electronic access to MS/MS peptide fragmentation spectra used to search databases in identifying proteins using our Scaffold software used in conjunction with the Proteous LIMS package.

**Protein Complex Analysis:** None gel based Protein identification utilising tandem ion exchange and reversed phase chromatography is now available.

**Intact Mass Analysis:** Accurate mass of purified Proteins in solution can be determined using our dedicated chip based chromatography system on the Q-TOF

**Post Translational Modification (PTM) Projects:** Low level modifications can be identified using combinations of ano-electrospray and MALDI mass spectrometers. Ubituqination and Sumolayed site determination are now included as well as phosphorylation, acetylation and disulphide bridge assignment.

**Quantitative Methods:** iTRAQ for Protein and label free quantitation for phosphorylated peptides are now available and we are currently investigating SILAC methodologies.

**2-D Gel Electrophoresis:** We can provide advice, equipment, reagents, and expertise for researchers to generate quality 2-D gel data. The laboratory provides LRI researchers with the opportunity to combine proteomic analyses with their own research. The laboratory has a range of specialist equipment to allow the reproducible running of 2-D gels, and scanning and software packages to enable analysis of 2-D gels.

Publications listed on page 136
The Protein Purification Facility (PPF) came into existence in 2008 as a new LRI facility that engages with research groups in projects concerning expression and purification of proteins using a variety of high-end protein expression technologies.

High quality purified proteins is a pre-requisite for a range of applications in the quest to elucidate the molecular origins of cancer. One such application is determination of 3D protein structures using x-ray crystallography. Another end-point application for proteins could be activity assays of a protein and mutant variants identified in cancers. The PPF interfaces closely with the four LRI structural groups and act as a project enabler for biologically oriented groups keen to pursue a structural determination of their proteins of interest or who have other end-point applications for high-grade protein.

Baculovirus
Baculovirus expression technology constitutes the backbone of PPF’s operations. It is an established technology that yields up to 10 mg protein/litre of insect cell culture. Numerous proteins, whose expression has proven difficult in E. coli, have been successfully produced.

During 2008, we streamlined the protocol for baculovirus generation and adopted a real-time PCR based method for titration of viral stocks. Developments in the viral packaging DNA have eliminated the need to clone single virus. These changes have reduced the time-scale for virus production considerably, hence increasing capacity and allowed a shift of our focus from virus generation to optimisation of protein expression.

Co-expression of multiple proteins by co-infection with multiple viruses is an evolving area of particular interest that allows isolation of protein complexes consisting of difficult-to-express individual proteins.

Mammalian expression
Certain proteins from higher eukaryotes require chaperones, some of which are absent from insect cells, to acquire their final fold. We are therefore implementing mammalian cells as expression hosts for such proteins. We use the glycosylation-deficient Lec8 cell-line, which produce proteins with homogenous N-glycans. Our Lec8 cell-lines are engineered to contain Flp-In™ recombination sites at transcriptionally active chromosome positions, ensuring that the stable Lec8 cell-lines are isogenic and produce proteins at consistently high levels.

Purification
The majority of the proteins are expressed with cleavable (in-house 3C or TEV protease) tags (6xHis or GST are frequent choices). Further ‘polishing’ is performed by standard chromatography methods such as gel filtration.

Future developments
In 2008, PPF interacted with more than 15 different LRI groups. We are actively seeking new collaborations and are planning the implementation of new activity assays and technologies to the benefit of the LRI community.
The Transgenic Service comprises a team of seven people who can provide the techniques to generate mice with a specifically altered genetic make up, allowing researchers to reveal gene function and to model disease in this important mammalian organism.

The Transgenic Service is based at Clare Hall. In 2008 a new Clare Hall facility, Poplar (P) block, opened providing, in addition to improved caging capacity for mice, a purpose built laboratory for the Transgenic Service.

**An outline of the services provided**

The techniques provided can be grouped into two categories, the techniques relevant to the production of genetically modified mice and those important in the maintenance of mice at Clare Hall, their health status and colony management.

As a brief summary, the techniques relevant to production are, mouse embryonic stem (ES) cell culture, derivation and transfection, microinjection of ES cells to generate chimeras or ES cell derived mice, diploid and tetraploid aggregation techniques, microinjection of cDNA or BAC into one-cell embryo (pronuclear injection) and in vitro fertilisation.

The techniques required for mouse colony maintenance are cryopreservation and rederivation. Cryopreservation providing a means for long term storage and distribution of mouse strains, either through collection of sperm, embryos or ovaries. 1600+ lines are stored, a valuable resource for those working in this field.

**Projects to extend and enhance the work of the service**

The techniques used to modify the mouse genome continue to evolve, it is important we provide an efficient and up to date service so a number of developmental projects are underway to trial and test new techniques.

There have been two recent and key publications in transgenic technology. Firstly, the production of ES cell derived mice from injection of 8 cell embryos, secondly, modifications to existing sperm freezing protocols to increase the applicability of this fast and efficient means of strain storage.

Both techniques have already been trialled successfully at Clare Hall. Both techniques are in routine use. These advances will allow us to complete projects more quickly, and reduce the numbers of mice required.
Paul Bates

Biomolecular Modeling Laboratory

Primary Research Papers

Facundo Batista

Lymphocyte Interaction Laboratory

Primary Research Papers

Other Publications
- Arana E, Harwood NE, Batista FD. Regulation of integrin activation through the B-cell receptor. J Cell Sci. 2008;121(14):2279-86
- Batista FD and Harwood NE. The who, how and where of antigen presentation to B cells. Nat Rev Immunol 2008;doi:10.1038/nri2454

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Axel Behrens

Mammalian Genetics Laboratory

Primary Research Papers

Dominique Bonnet

Haematopoietic Stem Cell Laboratory

Primary Research Papers

Other Publications
Bonnet D. In vivo evaluation of Leukemic Stem Cells through xenotransplantation model Curr Protoc Stem Cell Bio. 2008;Chapter 3:Unit 3.2

Julian Downward (page 28)
Signal Transduction Laboratory
Primary Research Papers

Other Publications
Cully M, Downward J. SnapShot: Ras Signaling. Cell 2008;133 (7):1292-1292

Holger Gerhardt (page 30)
Vascular Biology Laboratory
Primary Research Papers
Graupera M, Guillermet-Guibert J, Foukas LC, Phng LK,

Julia Cooper (page 26)
Telomere Biology Laboratory
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Other Publications

Other Publications


Other Publications

Caroline Hill (page 32)
Developmental Signalling Laboratory
Primary Research Papers

Daly AC, Randall RA, Hill CS. Transforming growth factor-induced Smad1/5 phosphorylation in epithelial cells is mediated by novel receptor complexes and is essential for anchorage-independent growth. Mol Cell Biol. 2008;28(22):6889-902

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Nancy Hogg (page 34)
Leukocyte Adhesion Laboratory
Primary Research Papers

David Ish-Horowicz (page 36)
Developmental Genetics Laboratory
Primary Research Papers


Banafshe Larijani (page 38)
Cell Biophysics Laboratory
Primary Research Papers


Other Publications


A developing Drosophila wing harbouring multiple tumours (pink). Actin is stained in green, Dig protein in blue.
Julian Lewis (page 40)

Vertebrate Development Laboratory

Primary Research Papers

Other Publications
Lewis J. From signals to patterns: space, time, and mathematics in developmental biology. Science. 2008;322(5900):399-403

Taija Makinen (page 42)

Lymphatic Development Laboratory

Primary Research Papers

Neil McDonald (page 44)

Structural Biology Laboratory

Primary Research Papers

Paul Nurse/ Jacqueline Hayles (page 46)

Cell Cycle Laboratory

Primary Research Papers

Peter Parker (page 48)

Protein Phosphorylation Laboratory

Primary Research Papers
Faisal A, Saurin A, Gregory B, Foxwell B, Parker PJ. The scaffold MyD88 acts to couple protein kinase C epsilon to

Immunofluorescence image showing cultured human lymphatic (green) and blood (red) endothelial cells.
Verma SK, Ganesan TS, Parker PJ. The tumour suppressor RASSF1A is a novel substrate of PKC. FEBS Lett. 2008;582(15):2270-6

Gordon Peters (page 50)
Molecular Oncology Laboratory

Primary Research Papers

Caetano Reis e Sousa (page 52)
Immunobiology Laboratory

Primary Research Papers

Research Publications 125
Erik Sahai (page 54)

Tumour Cell Biology Laboratory

Primary Research Papers

Other Publication

Giampietro Schiavo (page 56)

Molecular Neuropathobiology Laboratory

Primary Research Papers

Other Publication

Almut Schulze (page 58)

Gene Expression Analysis Laboratory

Primary Research Papers

Martin Singleton (page 60)

Macromolecular Structure and Function Laboratory

Primary Research Papers

Charles Swanton (page 62)

Translational Cancer Therapeutics Laboratory

Primary Research Publication
Other Publications

Nic Tapon (page 64)
Apoposis and Proliferation Control Laboratory
Primary Research Paper

Takashi Toda (page 68)
Cell Regulation Laboratory
Primary Research Papers

Other Publications

Ian Tomlinson (page 70)
Molecular and Population Genetics Laboratory
Primary Research Papers
Graham T, Halford S, Page KM,Tomlinson IP . Most low-level microsatellite instability in colorectal cancers can be explained without an elevated slippage rate. J Pathol. 2008;215(2):204-10


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Sharon Tooze (page 72)

Secretary Pathway Laboratory

Primary Research Papers


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Richard Treisman (page 74)

Transcription Laboratory

Primary Research Papers


Frank Uhlmann (page 76)

Chromosome Segregation Laboratory

Primary Research Papers


Other Publications


Michael Way (page 80)

Cell Motility Laboratory

Primary Research Papers


Nick Wright (page 82)

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Simon Boulton (page 84)

DNA Damage Response Laboratory
Primary Research Papers

False-coloured scanning electron micrograph of prostate cancer cells.

DNA Damage Response Laboratory
Primary Research Papers

Other Publications

False-coloured scanning electron micrograph of prostate cancer cells.
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Vincenzo Costanzo (page 86)
DNA Damage and Genomic Stability Laboratory

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John Diffley (page 88)
Chromosome Replication Laboratory

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Tim Hunt (page 90)
Cell Cycle Control

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Trenz K, Errico A, Costanzo V. Plxl is required for chromosomal DNA replication under stressful conditions. EMBO J. 2008;27(6):876-85

Other Publication
Hunt T. You never know: Cdk inhibitors as anti-cancer drugs Cell Cycle 2008;7(24):3789-3790

Immunofluorescence image of virus particles (purple) infecting Hela cells, actin cytoskeleton (green).

Drosophila larval wing imaginal disc with nuclei in blue and GFP (green) marking the antero-posterior boundary.
Peter Karran (page 92)

Mammalian DNA Repair Laboratory

Primary Research Papers

Other Publications

Tomas Lindahl (page 94)

Mutagenesis Laboratory

Primary Research Papers

Mark Petronczki (page 98)

Cell Division and Aneuploidy

Other Publications

Jesper Svejstrup (page 100)

Mechanisms of Gene Transcription

Primary Research Papers

Helle Ulrich (page 102)

DNA Damage Tolerance Laboratory

Primary Research Papers

Other Publications

Confocal image showing the cell-cell junctions (green) and actin cytoskeleton (red) of cultured lymphatic (blue) and blood endothelial cells.
Steven West  (page 104)
Genetic Recombination Laboratory

Primary Research Papers

Other Publications

Dale Wigley  (page 106)
Molecular Enzymology Laboratory

Primary Research Papers
Saikrishnan K, Griffiths SP, Cook N, Court R, Wigley DB. DNA binding to RecD2 of the 1B domain in SF1B helicase activity. EMBO J. 2008;27(16):2222-9

Electron Microscopy  (page 110)
Lucy Collinson

Primary Research Papers

Experimental Pathology  (page 112)
Gordon Stamp

Primary Research Papers
Fluorescence Activated Cell Sorter

Derek Davies

Primary Research Papers

Other Publications

High Throughput Screening

Mike Howell

Primary Research Papers

In Situ Hybridisation

Richard Poulsom

Primary Research Papers
Fang TC, Otto WR, Jeffery R, Hunt T, Alison MR, Cook HT, Wright NA, Poulsom R. Exogenous bone marrow cells do not rescue non-irradiated mice from acute renal tubular damage caused by HgCl2, despite establishment of chimaerism and cell proliferation in bone marrow and spleen. Cell Prolif. 2008; 41(4): 592-606


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Light Microscopy (page 116)

Daniel Zicha
Primary Research papers

Protein Analysis (page 117)
Mark Skehel (Clare Hall)
Primary Research papers

Other Research Publications
Bixel MG, Adams RH. Master and commander: continued expression of Prox1 prevents the dedifferentiation of lymphatic endothelial cells. Genes Dev. 2008;22(23):3232-5
Theses

Ditte Andersen
Tapon Laboratory

Emilie Bureau
Bonnet Laboratory
Investigation of the role of Haematopoetic Cell Kinase in and leukemic haematopoiesis (2008)

Sophie Pinner
Sahai Laboratory

Ofer Rog
Cooper Laboratory
Characterization and consequences of telomere replication gone awry (2008)

Christine Schmidt
Uhlmann Laboratory
Genomic analysis of cohesin dynamics in fission yeast (2008)

Stefania Segditsas
Tomlinson Laboratory
Mechanisms of intestinal tumorigenesis resulting from APC mutations (2008)

Yingdi Wang
Adams Laboratory
Regulation of the angiogenic growth of blood and lymphatic vessels by ephrin-B2 (2008)

Jordan Ward
Boulton Laboratory
Insight into homologous recombination at replication blocking lesions through analysis of the C.elegans RAD-51 paralog, RFS-1

Ina Weisswange
Way Laboratory
Analysis of Vaccinia virus actin tail nucleating complex dynamics (2008)
External Funding

Grants

Association for International Cancer Research
Bonnet Laboratory
Svejstrup Laboratory

Axonaltransp
Schiavo Laboratory

British Cancer Campaign
Boulton Laboratory
Sahai Laboratory

Celltech
Hogg Laboratory

European Commission
Bonnet Laboratory
Costanzo Laboratory
Diffley Laboratory
Hill Laboratory
Hunt Laboratory
Parker Laboratory
Reis e Sousa Laboratory
Schiavo Laboratory
Svejstrup Laboratory
Thompson Laboratory

Leukaemia Research Fund
Bonnet Laboratory
Tomlinson Laboratory
West Laboratory

European Molecular Biology Organisation
Batista Laboratory
Behrens Laboratory
Boulton Laboratory
Costanzo Laboratory
Gerhardt Laboratory
Tapon Laboratory

Fanconi Anaemia Research Fund
Boulton Laboratory

Human Frontier Science Programme
Uhlmann Laboratory

Lister
Costanzo Laboratory

Louis-Jeantet
West Laboratory

Medical Research Council
Bonnet Laboratory
Swanton Laboratory

Wellcome
Schiavo Laboratory

Postdoctoral Fellowships

CJ Martin Fellowship
Andrew Deans – West Laboratory

Department of Health
Elinor Sawyer – Tomlinson Laboratory

Deutsche Forschungsgemeinschaft
Thomas Wechsler - West Laboratory

Dutch Cancer Society
Elza De Bruin – Downward Laboratory

European Commission – Marie Curie
Eleonora Agricola – Hill Laboratory
Eva Grönroos – Hill Laboratory
Carola Langer – Treisman Laboratory
Sandra Lopez-Aviles – Uhlmann Laboratory
Maria Ocampo-Hafalla – Uhlmann Laboratory
Rocio Sancho – Behrens Laboratory
Cristina Aguilera – Behrens Laboratory
David Sancho-Madrid – Reis e Sousa Laboratory
Lena Svensson – Hogg Laboratory
Electron Microscopy of B Lymphocytes (rounded) binding antigen on the surface of an antigen presenting cell.
Graduate Students
At any one time, the LRI has approximately 100 graduate students at different stages of their 4-year PhD programme. The students contribute to the international flavour of our Institute – at present about 30% of our students are British, 40% are from elsewhere in the EU and 30% come from further afield including North and South America, China, Japan and India. LRI students are registered for their PhDs at University College London (UCL) and most are funded by Cancer Research UK LRI Studentships.

The LRI Graduate Student Programme
The LRI Graduate Student Programme starts each September with 3 induction days to give students a head start in getting to know the LRI, its staff and each other. This year, sessions included keeping on top of the literature, organising your lab work and lab book, getting the most out of journal clubs and experiment design. At the end, the new students were joined by the rest of the Institute to hear Andreas Pichlmair, a former student in Caetano Reis e Sousa’s lab, present the work that won him the 2007 Pontecorvo Prize for the best CRUK-funded PhD thesis.

Other key points in the student calendar included the 10 minute talks, which students give at the end of their first term, annual reports and thesis committee meetings, and the report, seminar and thesis committee meetings that precede the upgrade of second year students’ registration from MPhil to PhD. The second year students voted on the best Upgrade Seminar and the Cambridge Bioscience-sponsored Student Upgrade Prizes were awarded to Alice Genevet (Lincoln’s Inn Fields), Ozan Aygun (Clare Hall) and Guillermo Menendez (Lincoln’s Inn Fields). These students presented their work for a second time in an open seminar to the Institute, which was followed by a student-organised Upgrade Ball for PhD and summer students.

Each year our students participate in a joint student conference with other European cancer research institutes. The 2008 conference, organised and hosted by students from the Netherlands Cancer Institute (NKI) in Amsterdam, was a great success in allowing students to present their work and talk to each other about their research and scientific life in different research institutes across Europe. 20 LRI students attended, together with students from across the rest of CRUK, the European School of Molecular Medicine (SEMM), Milan, Italy and the Spanish National Cancer Centre (CNIO), Madrid Spain. In addition, all our third year students attended the annual National Cancer Research Institute Conference in Birmingham, many students attended the LRI retreat in April, and many presented their work at specialist scientific conferences.

Graduate Student Support and Training
As well as being exciting and rewarding, doing a PhD can be a challenging and demanding time. To help with this, all LRI students have access to an extensive support network to provide advice, guidance and training. The main source of academic guidance is the primary supervisor and students also receive training from other lab members, other LRI research and research service labs. In addition, each student has a thesis committee, made up of 2 additional group leaders, which they meet with at key points during their PhD.
Other sources of support are the LRI Academic Director, Sally Leevers, and the Research Manager for Graduate Students, Erin Fortin, who run the LRI graduate student programme. The Graduate Student Advisors (Simon Boulton, Nancy Hogg, Taija Makinen, Gipi Schiavo, Jesper Svejstrup and Michael Way) are always available to provide friendly and confidential advice for students. The 2008 intake of students also benefited from each having an assigned student buddy, for them to chat to informally about getting settled in the LRI and embarking on their PhD.

In addition to training in practical research skills, a range of courses are available to LRI students provided in house by the LRI Trainer, David Bacon, and the Academic Director, by CRUK, and by the UCL graduate school. These include courses on presenting and publishing research, thesis and report writing, and communicating research to a non-specialist audience.

**Postdoctoral Fellows**

There are usually approximately 200 postdocs working at the LRI, funded by competitively awarded postdoctoral fellowships from the LRI, the European Molecular Biology Organisation, the Human Frontiers Science Programme, the Federation of European Biochemical Societies, the Royal Society, Marie Curie and various other sources.

Throughout 2008, the postdocs got together at a number of forums to get to know each other and discuss issues concerning them. They also organised the first one-day LRI postdoc retreat, with a theme of ‘Collaboration, Community and Communication in Science’. The focus of the day was a research speed dating style event where postdocs had 9 minutes to design a collaborative project with another postdoc, before repeating the process 5 more times! It was frantic and fun, the quality of the devised projects was extremely high, and the overall winners were Anja Hanisch and Rocio Sanchez for their project using zebrafish as a model organism to study kinases. The other highlights were the inspiring talks from Professor Sir David Lane and Dr Jonathon Pines, which provided fascinating and humorous insights into their experiences forming successful collaborations, and emphasised the benefit of being open and generous with ideas, data and reagents.

Another new activity for the postdocs was a series of careers talks providing advice on how to establish themselves as independent researchers. There was an initial series of discussion sessions, culminating in an afternoon workshop on applying for Career Development Awards, with talks from Matthew Wakelin, a senior research manager for the CRUK Science Funding team, and ex-LRI postdocs holding these awards. These were followed by five more practical sessions covering all aspects of the application process, from the preparing the initial cover letter and CV to giving a job seminar and being interviewed.

**The LRI Summer Student Programme**

2008 was the second year of the LRI Summer Student Programme, in which penultimate year undergraduate students are paired with postdoctoral supervisors for the summer. The programme gives undergraduates research experience to help them decide whether they would like to do a PhD, and gives LRI postdocs formal interview and supervision experience. Postdocs submitted short project descriptions to join the programme, then interviewed the shortlisted applicants and decided on the successful applicants. 18 summer students started their 10 week programme in June, with a 1 day induction, introducing them to the LRI and laboratory work. Over the summer they were given advice on choosing and applying for PhD positions, and on presenting their research. The programme culminated in a Summer Student Symposium, at which each student gave a short talk about their work over the summer. The talks were impressive – it was clear that the students had gained valuable practical experience, and developed a good
Seminars and Conferences

Special Seminars 2008

January
Rick Firtel
University of California San Diego
Spatio-temporal control of chemotaxis via Ras signaling networks

February
Marteen Van Lohuizen
The Netherlands Cancer Institute, Division of Molecular Genetics
Polycomb repressors controlling stem cell fate: Implications for cancer and development

Cornelis Weijer
Wellcome Trust Biocentre, University of Dundee
Chemotactic cell migration during development

March
Eric Wieschaus
HHMI/Princeton University
Patterning gene expression and cell shape change in the drosophila embryo

Elena Conti
Max Planck Institute for Biochemistry
Molecular mechanisms of RNA degradation

April
Anton Berns
The Netherlands Cancer Institute, Dept. of Molecular Genetics
Mouse models for human cancer

May
Jennifer Doudna
University of California, Berkeley
Hijacking the ribosome: Translational control by viruses and cells

Wieland Huttner
Max Planck Institute of Molecular Cell Biology and Genetics
The cell biology of neural stem and progenitor cells

June
David Pellman
Dana-Farber Cancer Institute
Polyplody, aneuploidy, and genetic instability

Celeste Simon
Howard Hughes Medical Institute and Abramson Family Cancer Research Institute
Hypoxia, stem cells, and tumor progression

July
Gerard Evan
University of California, San Francisco
Modelling cancer therapies in the mouse

November
Bruce Stillman
Cold Spring Harbour Laboratory
A role for ORC in chromosome and centrosome duplication

December
Jeff Settleman
Harvard Medical School
Oncogene ‘addiction’ and therapeutic opportunities in human cancer

Drosophila eye-antennal disc with nuclei in blues and Wingless/Wnt expression in red.
Conferences 2008

29 February
Postdoc Retreat

This year saw the inaugural LRI Postdoc Retreat organised by the postdocs themselves. The chosen theme for this first meeting was ‘Collaboration, Community and Communication in Science’. Professor Sir David Lane and Dr Jonathon Pines gave two inspirational talks on their scientific careers. The Science ‘speed-dating’ game fostered collaborations between research fellows on both sites.

28-30 April
LRI Retreat

The first LRI Retreat took place at the University of Kent, with over 350 attending, including representatives from all the research and service groups based at the Institute, actively participating throughout the three days. The Retreat provided a unique opportunity for groups, from both sites of the Institute, to get together and present their work in an informal and relaxed environment.

A full programme saw half of the Research Groups presenting talks, often consisting of several presentations from members of the laboratory, while the remaining laboratories and service labs presented their work during two poster sessions. The keynote speech was given by Tomas Lindahl, from the Clare Hall Laboratories, who is due to retire in 2009. The success of this event has secured its place in the 2009 calendar where the lists of research groups presenting posters and talks will be reversed.

28-30 May
LRI Symposium on Chromosome Biology

The theme for the second LRI Symposium was Chromosome Biology, a topic for which there is a long established interest at the London Research Institute. The organisers: Simon Boulton, Julie Cooper and Frank Uhlmann, brought together 27 internationally renowned speakers in the field speaking on a wide array of topics, giving a snapshot of this dynamic field and where it may go in the future as technology develops.

There were six sessions; Chromosome Replication, Views of Chromosomes, Chromosome Architecture, Mitotic Chromosomes, each sparking lively debates that contributed to make a hugely successful conference enjoyed by everyone who attended.

11-13 June
Graduate Student Conference

20 students represented the London Research Institute at the Graduate Student Conference. The 2008 event was hosted by the Netherlands Cancer Research Institute (NKI) and was attended by students from throughout CR-UK and other research institutes including the Vienna Biocenter, and European School of Molecular Medicine in Milan. This meeting provided an opportunity to network with fellow students at top European cancer institutes and discuss their research experiences with their international peers.

Dates for 2009

13 February
Postdoc Retreat

16-18 March
LRI Retreat

3-5 June
Graduate Student Conference
Organised by LRI student

17-19 June
LRI Symposium
Developmental Biology: Organising tissues in time and space
Administration

LRI administration
The LRI Administration team provides the Director with the administrative infrastructure and support to ensure the smooth running of the Institute. The team led by the LRI Director of Operations are responsible for the academic infrastructure through the administration of the academic committees for students and postdocs, management of LRI Technology Core Facilities, finance, co-ordinating Institute wide initiatives and providing general administrative support to the Research Laboratories.

Graduate Student Administration
Twenty-two graduate students and one clinical research-training fellow started at the LRI in September 2008. This year also saw 21 undergraduates join the 10-12 week LRI Summer Student Programme. Management of the graduate students recruitment process has been transformed using our in house developed FileMaker Pro Recruitment Database.

Specialised Training Programme
In 2008 a new software training programme was introduced to help students achieve maximum efficiency and save time, allowing them to gain confidence using the applications they will need throughout their careers. Courses are available for various applications, and are tailored to suit student needs. Classes are held in small groups of five or, if necessary, with one to one support.

Postdoctoral Fellows Administration
In 2008 six Fellowships Committee meetings were held to approve funding for Postdoctoral Fellowships.

102 applications were made to the committee with 76% of those applications being funded.

The Postdoc Forum, organised by postdocs for postdocs, continued to hold regular meetings, and a successful Postdoc Retreat on the subject of Collaboration, Community and Communication in Science was held in London in February.

Administrative Support for Group Leaders
Group leaders receive comprehensive secretarial and administrative support from research administrators. Members of the team have been involved in reviewing and revising standard operating procedures and improving the intranet resources used by all the administrators to help them execute their responsibilities.

Laboratory Management Services
The Laboratory Services and Support team liaises closely with the research and service laboratories at the LRI. The team also includes the Electronics Department and Fly Facility services. We facilitate the design, construction, refurbishment and equipping of laboratory space and deal with all communal equipment and service contracts. In 2008 we oversaw the refurbishment of a number communal equipment rooms and begun refurbishment of the aquarium facilities. This year saw an expansion of the Clare Hall campus with the opening of the Poplar Building the refurbishment of Cedar Block. Building work was begun on the new Willow Block.
Institute Management

London Research Institute Management Committee

Richard Treisman PhD FRS (Chair)
Julian Downward PhD FRS
John Diffley PhD FRS
Caroline Hill PhD
David Ish-Horowicz PhD FRS
Nigel Peat MSc
Stephen West PhD FRS
Ava Yeo PhD

London Research Institute Faculty Committee

Richard Treisman PhD FRS (Chair)
David Ish-Horowicz PhD FRS (Co-chair)
Dominique Bonnet PhD
Simon Boulton PhD
Julie Promisel Cooper PhD
Julian Lewis PhD
Caetano Reis e Sousa PhD
Frank Uhlmann PhD
Helle Ulrich PhD
Michael Way PhD
Ava Yeo PhD (in attendance)
Julian Downward PhD FRS (ex officio)
John Diffley PhD FRS (ex officio)

London Research Institute Fellowships Committee

Peter Parker PhD FRS (Acting Chair until June 2008)
David Ish-Horowicz PhD FRS (Chair from June 2008)
Dominique Bonnet PhD
Vincenzo Costa PhD (from January 2008)
Caroline Hill PhD (from January 2008)
Svend Petersen-Mahrt PhD
Giampietro Schiavo PhD
Barry Thompson PhD (from March 2008)
Helen Walden PhD
Richard Treisman PhD FRS (ex officio)
John Diffley PhD FRS (ex officio)
Sally Leevers PhD (in attendance)
Ava Yeo PhD (in attendance)

London Research Institute Graduate Students Advisors Committee

Sally Leevers PhD (Chair)
Ralf Adams PhD (until May 2008)
Simon Boulton PhD
Nancy Hogg PhD (until December 2008)
Taija Makinen PhD (from September 2008)
Gordon Peters PhD (from December 2008)
Giampietro Schiavo PhD
Jesper Svejstrup PhD
Sharon Tooze PhD (until September 2008)
Michael Way PhD (from September 2008)
Professor Christopher Danpure PhD (UCL)
Ava Yeo PhD (in attendance)
Sabina Ebbols (in attendance from November 2008)
Erin Fortin MPhil (in attendance)
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